T.C.

YEDİTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

PROTECTIVE EFFECT OF WHEY PROTEIN AND N-ACETYLCYSTEINE AGAINST CISPLATIN-INDUCED NEPHROTOXICITY

DOCTOR OF PHILOSOPHY THESIS

YAZAN AWAD, Pharm.D, MSc

ISTANBUL 2024

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DECLARATION

I hereby declare that this thesis, titled “Protective Effect of Whey Protein and N-Acetylcysteine Against Cisplatin-induced Nephrotoxicity," is the result of my own original research and work. All sources of information and data have been duly acknowledged and cited appropriately.

I affirm that this thesis has not been submitted, either in whole or in part, for any degree or diploma at any other institution, and it is solely submitted in fulfilment of the requirements for the degree of PhD in Pharmaceutical Toxicology at Yeditepe University.

I confirm that I have followed the university's guidelines and policies regarding research ethics and academic integrity in the preparation and submission of this thesis.

Yazan Awad

DEDICATION

To my beloved family, whose unwavering love, support, and encouragement have been the foundation of my success.

To my father, Dr. Waddah Awad, for his steadfast belief in me and his constant support.

To my mother, Lawyer Fayzeh Alhelo Dit Aljamal, whose dedication and love have been my greatest source of strength.

To my sister, MSc. Malda Awad, my friend and companion throughout this journey.

And to my loved ones, who have been my source of inspiration and motivation.

This thesis is dedicated to you all.

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Abbreviations

APAP	Acetaminophen
CAT	Catalase
CDDP	cis-diaminedichloroplatinum II
CP	Cisplatin
CTR1	copper transporter 1
CTR2	copper transporter 2
FDA	Food and Drug Administration
GFR	Glomerular filtration rate
GSH	Glutathione
GSH-PX	Glutathione peroxidase
LPO	Lipid peroxidation
MDA	Malondialdehyde
NAC	N-Acetylcysteine
NAPQI	N-acetyl-p-benzoquinone imine
NSCLC	Non-small cell lung cancer
OCT	Organic cation transporters
ROS	Reactive oxygen species
SCLC	Small cell lung cancer
SDS	sodium dodecyl sulphate
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
TBA	thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TNF-α	Tumor necrosis factor α

ABSTRACT

Cisplatin is a renowned chemotherapy agent used to manage several types of cancer. However, its strong nephrotoxic and hepatotoxic side effects restrict its clinical application. This research aims to investigate the potential nephroprotective and hepatoprotective impact of whey protein and N-acetylcysteine (NAC) in reducing the toxic effects of cisplatin on the kidney and liver. We induced nephrotoxicity and hepatotoxicity in a rat model by administering cisplatin, with pre-and post-treatment using whey protein and NAC. Important parameters were measured, such as the biochemical markers alanine aminotransferase, aspartate aminotransferase, urea, and creatinine, as well as the oxidative stress marker malondialdehyde and the antioxidant enzymes catalase and glutathione peroxidase. Moreover, histopathological analysis was performed to assess tissue injury. The outcomes revealed that whey protein and NAC effectively reduce cisplatin-induced liver and kidney damage. The strong antioxidant characteristics of whey protein reduced oxidative stress, and NAC increased antioxidant enzyme activity. Compared to the cisplatin-only group, biochemical parameters indicated improved liver and kidney function in the therapy groups. Histopathological examination showed less tissue damage, confirming the benefits of NAC and whey protein as nephroprotective agents. These results imply that the co-administration of whey protein and NAC presents a viable synergistic therapeutic strategy to guard against hepatotoxicity and nephrotoxicity caused by cisplatin, hence improving its clinical utility in cancer treatment.

Keywords: Cisplatin, Whey Protein, N-acetylcysteine, Nephrotoxicity, Hepatotoxicity, Chemotherapy, Cancer

ÖZET

Yaygın olarak kullanılan kemoterapi ilacı sisplatin'in güçlü nefrotoksik ve hepatotoksik yan etkileri klinik uygulamasını kısıtlar. Bu araştırma, whey (peynir altı suyu) proteininin ve N-asetilsisteinin, sisplatinin böbrek ve karaciğer üzerindeki toksik etkisini azaltmadaki potansiyel nefroprotektif ve hepatoprotektif etkisini incelemeyi amaçlamaktadır. Whey protein ve NAC ile tedavi öncesi ve sonrası ile ilişkili sisplatin uygulayarak bir sıçan modelinde nefrotoksisite ve hepatotoksisiteyi indükledik. Biyokimyasal belirteçler alanin aminotransferaz, aspartat aminotransferaz, üre ve kreatinin yanı sıra oksidatif stres belirteci malondialdehit ve antioksidan enzimler katalaz ve glutatyon peroksidaz gibi önemli parametreler ölçüldü. Ayrıca, doku hasarını değerlendirmek için histopatolojik analiz yapıldı. Sonuçlar, whey proteinin ve N-asetilsisteinin, sisplatinin neden olduğu karaciğer ve böbrek hasarını etkili bir şekilde azalttığını ortaya koydu. Whey proteininin güçlü antioksidan özellikleri oksidatif stresi azalttı ve antioksidan enzim aktivitesi NAC tarafından artırıldı. Sadece sisplatin grubuna kıyasla, biyokimyasal parametreler tedavi gruplarında karaciğer ve böbrek fonksiyonunun iyileştiğini gösterdi. Histopatolojik inceleme, NAC ve whey proteininin koruyucu olarak faydalarını doğrulayan daha az doku hasarı gösterdi. Bu sonuçlar, whey proteini ve NAC'nin birlikte uygulanmasının, sisplatinin getirdiği hepatotoksisiteye ve nefrotoksisiteye karşı korunmak için uygulanabilir bir sinerjik terapötik strateji sunduğunu ve dolayısıyla kanser tedavisinde sisplatinin klinik faydasını iyileştirdiğini göstermektedir.

Anahtar kelimeler: Sisplatin, Peynir altı suyu proteini, N-asetilsistein, Nefrotoksisite, Hepatoksisite, Kemoterapi, Kanser,

1. Introduction

As the second leading cause of mortality within the US, cancer remains one of the enormous worldwide health troubles [1]. The last pandemic of severe acute respiratory syndrome COVID-19 (SARS-COV-2) impacted the detection and treatment of cancer in 2020. Limited access to scientific offerings because of healthcare facility saturation and panic about COVID-19 exposure brought about delays in detection and remedy, which may also result in a brief-term decline in the prevalence of cancer accompanied by the aid of an upward thrust in superior-degree sickness and, in the end, higher loss of life [2]. However, due to the lag in disseminating population-based surveillance data, measuring all of these and other secondary effects associated with the pandemic at the population level will take several years [3]. In 2020, the number of new registered cases in Turkey was 233,834 while the number of deaths was 126,335 for both genders according to the International Agency for Research on Cancer [4]. Unlike the global incidence of cancer, lung cancer was the most common cancer among men in Turkey. On the other hand, breast cancer had the highest percentage among women.

The term cancer is used to refer to a disorder in the cellular division which is uncontrolled and might affect any organ or cell type in the body [5]. Several terms can be used to describe this disease such as malignant tumours or neoplasms. One distinguishing characteristic of cancer is the quick development of aberrant cells that quickly outgrow their typical bounds and can infiltrate nearby body sections before metastasizing to other organs [6]. The main reason for dying from cancer is extensive metastatic tumours [5].

Cancer is a very complex and considered as a serious health condition. However, it can be cured depending on many factors such as the type, stage and location of the malignancy itself. There are many choices or types of therapies that might be applied to treat cancer patients such as surgery, chemotherapy, and radiation therapy, which are traditional treatments. There are modern forms of therapy such as immunotherapy [7], targeted therapy [8], hormone therapy [9], gene therapy [10] and photodynamic therapy [11].

Cisplatin (CP), also referred as cis-diaminedichloroplatinum II (CDDP), is commonly administrated with other platinum-based chemotherapy agents in the management of diverse cancers [12]. However, the therapeutic application of CDDP is limited due to its high degree of toxicity, particularly concerning nephrotoxicity [13]. The administration of CDDP has been found to lead to nephrotoxicity in more than 25% of individuals due to its strong propensity to accumulate in the epithelial cells of renal proximal tubules [14]. The cytotoxic effects of cisplatin have been linked to many mechanisms, including direct DNA destruction, disruption of mitochondrial function, and induction of the apoptotic pathway [15]. The nephrotoxicity of cisplatin is modulated by the generation of reactive oxygen species (ROS) and/or the inhibition of the antioxidant defence system [16]. The therapeutic effectiveness of cisplatin can be augmented through the implementation of an adjuvant therapy that specifically targets the reduction of its detrimental side effects. Numerous investigations have provided evidence supporting the preventive effects of pharmacological medicines that inhibit the generation of ROS [17 - 19]. The adverse effect of cisplatin on the proximal tubule in rats has been reported frequently in the literature records [20 - 25]. As a consequence, the kidney injury is resemblance to that caused by ischemia [26 – 27]. Cisplatin-induced acute renal failure is frequently characterised by various indicators such as a significant decrease in glomerular filtration rate (GFR) [28], a diverse reduction in renal blood flow [26 – 27], a decline in urinary concentrating ability, and alterations in urine volume and creatinine clearance [21]. The recognition of effective approaches to mitigate or reduce the negative outcomes of cisplatin is of paramount significance.

The sulfhydryl donor N-acetylcysteine (NAC) has a variety of medicinal uses. Free radical scavenger, mitochondrial protector, and inhibitor of lipid peroxidation (LPO) and cellular necrosis are all roles it has been shown to play [20]. By preventing the biotransformation of xenobiotics, NAC also aids in liver detoxification [21]. By functioning as a precursor in the glutathione (GSH) production pathway, it improves a variety of cellular defense systems and increases the level of GSH within the cell [22]. Additionally, NAC has been widely applied as an efficient antioxidant against oxidative stress both in vivo and in vitro, and is capable of restoring a prooxidant/antioxidant balance that has been disrupted. [29 – 32] . It has been shown that the level of protection of Cisplatin nephrotoxicity in rats is dependent on the route of NAC administration and that only intravenous but not oral or i.p. administration was effective in protecting against CP-nephrotoxicity [31]. NAC was also shown to improve renal blood flow in acute renal failure produced by inferior vena cava occlusion (Conesa et al., 2001). Although a protective effect of NAC in CP-induced renal failure has been reported before [32 – 33].

Whey protein is comprised of various biologically active fractions, such as glycomacropeptide, β-lactoglobulin, α-lactalbumin, and lactoferrin, which have been associated with numerous health benefits in relation to cancer, infection, and inflammation [35]. A structural analysis has been suggested that the protein maintains its conformation following the binding of cisplatin [36]. According to an additional investigation, it has been proposed that lactoferrin exhibits a defensive impact against kidney injury caused by cisplatin. The observed protective mechanism of lactoferrin entails the mitigation of cisplatin accumulation within the renal system [37]. To date, no scholarly investigation has been conducted to examine the potential nephroprotective effects of whey protein in mitigating cisplatin-induced nephrotoxicity. Therefore, This research aims to evaluate the nephroprotective effect of whey protein and NAC in terms of cisplatin-induced nephrotoxicity.

1.1. Cisplatin

1.1.1. Historical Overview of Cisplatin

The compound, cisplatin, was initially synthesized by Michele Peyrone in the year 1845 [1], thus earning the nomenclature of Peyrone's salt for an extended duration. The structural elucidation of Peyrone's salt was accurately determined by Alfred Werner in the year 1893 [38]. However, the fortuitous revelation of the intriguing phenomenon of cellular division inhibition was serendipitously brought to light by Barnett Rosenberg, a distinguished biophysicist engrossed in the investigation of the ramifications of electric fields on bacterial proliferation. During his meticulous experimentation, platinum was employed as the electrode, while ammonium chloride served as the buffer medium. During the course of his experimental investigations, it was observed that the E-coli bacteria exhibited a notable phenomenon wherein their cellular dimensions expanded by a factor of 2 to 300 times beyond their customary size, rather than undergoing conventional cell division, upon the application of an electric field. Intriguingly, upon the cessation of the electrical field, the bacterial cells resumed their typical division process. While initially postulating that the electrical field played a pivotal role in regulating cell division, the researcher ultimately demonstrated that the inhibition of cell division was attributed to the liberation of a platinum compound from the electrode [39].

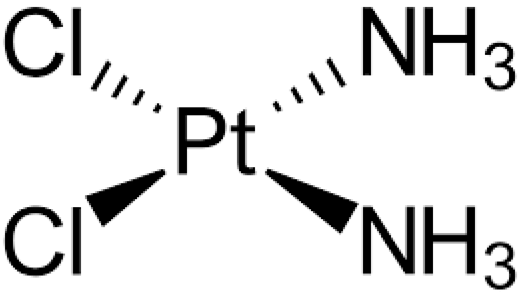

Figure 1 Chemical Structure of Cisplatin

In the year 1969, Rosenberg conducted a study wherein it was observed that cisplatin exhibited inhibitory effects on sarcoma 180 and leukemia L1210 in mice [40]. The subsequent examinations conducted on the pharmaceutical compound have demonstrated its efficacy in combating a diverse range of neoplastic conditions observed in various animal species [41]. The desired outcomes prompted the initiation of preclinical pharmacology and toxicology evaluations by esteemed institutions such as the National Cancer Institute (US) and the Wadley Institutes of Molecular Medicine [1]. In 1971, the National Cancer Institute initiated trial 1, which subsequently received approval from the US Food and Drug Administration (FDA) in 1978 for the treatment of testicular and ovarian cancer. In 1979, the United Kingdom also approved its use [1]. Figure 2 illustrates the significant milestones of the pharmacological development and utilization of cisplatin.

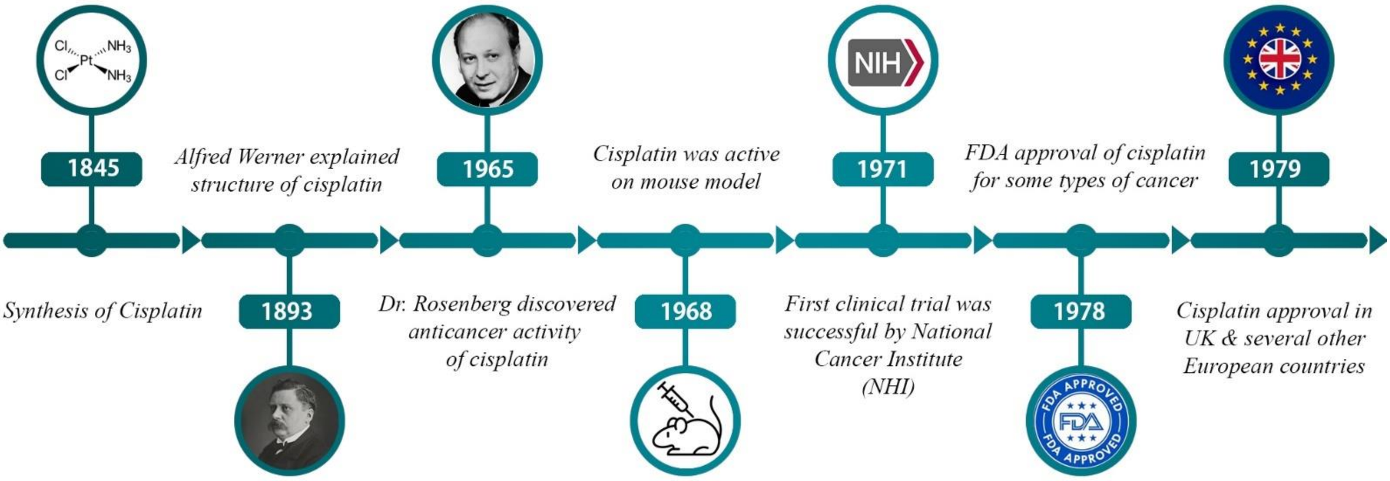

Figure 2 Milestones of Cisplatin

1.1.2. Mechanism of Action of Cisplatin

There are many mechanisms for the anticancer activity of cisplatin. However, the primary mechanism of action is binding to DNA as a major target [42].

Upon entry into the cell, cisplatin is activated in the cytoplasm. This process involves the replacement of chloride ions by water molecules, leading to the creation of a hydrolyzed derivative with enhanced electrophilic reactivity. This active form tends to interact with different nucleophilic groups, such as sulfhydryl groups in proteins and nitrogen donors in nucleic acids. Cisplatin has strong affinity for the N7 reactive site on purine residues that makes it highly effective at causing DNA damage in cancer cells, inactivation of GHS

disrupting cell growth and leading to programmed cell death. The most common changes in DNA structure that occur are the development of 1,2-intrastrand cross-links, including involving deoxyguanosine-deoxyguanosine d(GpG) and deoxyadenosine-deoxyguanosine d(ApG) adducts. The majority of these intrastrand adducts highlight the considerable influence of cisplatin-induced DNA alterations. Furthermore, the creation of several adducts, including 1,3-intrastrand deoxyguanosine-deoxyguanosine d(GpXpG) adducts and inter-strand crosslinks, in addition to nonfunctional adducts, enhances the cytotoxic effectiveness of cisplatin. Many studies have shown that DNA is a crucial target for cisplatin-induced cell death. Evidence suggests that cells with impaired DNA repair mechanisms are more susceptible to damage caused by cisplatin [43,44]. Figure 3 illustrates the cross-linking complexes of cisplatin on DNA.

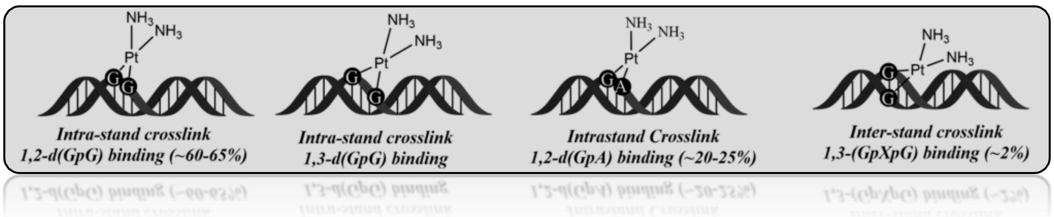

Figure 3 Cross-linking complexes of cisplatin on DNA

1.2. Clinical Indications of Cisplatin Chemotherapy

Lung cancer

It has a huge impact on public health worldwide because it is a severe widespread disease [45]. There are two main subtypes of the disease: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). There are differences in their unique growth and metastatic features. Small cell lung cancers (SCLCs) show aggressive behavior and rapid growth manner. The treatment of this type of cancer is associated with many challenges in order to be treated because they have a high tendency of metastasis among different organs [46]. During the management of SCLC, chemotherapy is considered the most appropriate therapeutical choice. Cisplatin and carboplatin are the key components in chemotherapy protocols. Cisplatin has a very powerful anticancer activity that frequently outweighs its side effects making it the preferred choice over carboplatin which includes renal toxicity and gastrointestinal complications, [47]. On the other hand, the treatment of NSCLC usually requires a comprehensive strategy tailored to the specific stage of the illness. Surgical resection is often used for localized illness in stages I and II, followed by adjuvant cisplatin-based chemotherapy to lower the chance of recurrence. Recent studies, such as the Lung Adjuvant Cisplatin Evaluation program, have highlighted the substantial survival advantage linked to adjuvant chemotherapy, demonstrating its effectiveness in enhancing patient outcomes [48]. Moreover, the discovery of new therapeutic targets has shown CD133 as a possible indicator for cancer-initiating cells in NSCLC, showing a higher CD133þESAþ population in primary NSCLC compared to normal lung tissue [49]. The progress demonstrates the changing nature of lung cancer treatment and the continuous search for enhanced therapeutic approaches. Although advancements have been made in comprehending the disease mechanisms and creating specific medicines, lung cancer still poses a significant challenge. Therefore, ongoing research is crucial to improve treatment results and reduce the impact of this severe illness.

Ovarian cancer

Among gynecological malignancies, ovarian cancer has the highest rate of mortality because it is detected at advanced stages most of the time due to inadequate screening methods and the absence of signs and symptoms in early stages. The usual or typical treatment at later stages includes surgical removal followed by a combination of platinum and taxane chemotherapy which is effective at first but recurrence occurs in about 75% of cases [50]. Resistance to chemotherapy develops in recurring cases, ultimately resulting in death. Although the cause of around 90% of ovarian carcinomas is unknown, a small portion is linked to genetic predisposition or has connections to breast and colon cancers [51]. Cisplatin derivatives are still widely used in ovarian cancer treatment despite their negative effects and the development of resistance. They are often combined with other substances like honey venom [52], withaferin [53], trichostatin A and 5-aza-2'-deoxycytidine [54], to address chemotherapy resistance in both sensitive and resistant ovarian cancer cells.

Head and neck carcinoma

Every year more than 600,000 new cases are reported internationally with head and neck squamous cell carcinoma which is considered as one of the most common types of cancer [54]. Although there have been improvements in therapeutical methods such as surgery, radiotherapy and chemotherapy. Head and neck squamous cell carcinoma still has a high rate of mortality. The consistent 5-year survival rate of around 50% has shown a slight decline in recent years. Cisplatin monotherapy is not effective in treating HNSCC. There is a previous study compared the effectiveness of cisplatin alone versus its combination with methotrexate, vinblastine, doxorubicin, and gemcitabine in patients who have been diagnosed with metastatic urothelial cancer [56,57].

Testicular cancer

Seminoma and non-seminoma are major kinds of testicular cancer that mainly impact young guys. Seminomas, found in individuals of all age groups, proliferate and spread at a slower rate compared to non-seminomas. Seminomas are mainly treated with cisplatin-based regimens, resulting in around 85% of advanced cases going into remission after three or four cycles of this therapy [1]. On the other hand, the usage of carboplatin alone shows a remission rate of 59% [58]. Non-seminomas are more common in males aged between late teens and early 30s rather than females. This type of cancer consists of four primary subtypes (embryonal carcinoma, yolk sac carcinoma, choriocarcinoma and teratoma). Teratoma patients achieve the highest benefits from a combination therapy that includes bleomycin, etoposide and cisplatin. The cure rates reach 90% and above [59]. Cisplatin was approved by the FDA in 1978 for the treatment of metastatic ovarian and testicular cancer [60]. Although the exact mechanism by which cisplatin is effective against testicular cancer is not fully understood, other possibilities have been suggested. Gong et al. emphasized the increased expression of Kindlin-2 in prostate cancer cells, which controls cancer cell death [61]. Usanova et al. found that a lack of interstrand-crosslink repair and reduced ERCC1-XPF expression are factors that make testis tumor cells more sensitive to cisplatin [62]. Another research showed that the presence of wild-type p53 protein, increased Oct4 levels, higher levels of proapoptotic Noxa protein, and lower levels of anti-apoptotic p21 protein in the cytoplasm are essential for testicular cancer cells to respond to cisplatin [63]. A different study found that high-mobility group box protein 4 (HMGB4), which is mainly found in testes, hinders the removal of cisplatin-DNA adducts, particularly 1,2-intrastrand cross-links [64]. This action increases the effectiveness of cisplatin therapy in testicular germ cell tumors.

Other types of cancer

Cisplatin has diverse applications outside its traditional role in the treatment of testicular, ovarian, and lung malignancies. It is effective for treating recurring childhood brain tumors [65], GIT cancer [66, 67] and leukemia among other conditions [68]. Cisplatin has shown considerable advantages in treating breast cancer by improving patient survival rates [69]. Cisplatin is also a highly effective chemotherapeutic medication used to treat several types of malignancies such as cervical, prostate, bladder, lung, and refractory non-Hodgkin's lymphomas. In glioblastoma multiforme (GBM), cisplatin is used in combination therapy with surgery, radiation, and temozolomide administration. Although survival rates have slightly improved, especially noticeable at the 5-year point, the median survival increase is still quite small at 2.5 months [70,71].

1.3. The role of cisplatin in oxidative stress induction

Day after day, oxidative stress was taking the attention of scientists as an essential factor of cisplatin-induced nephrotoxicity [72]. During the administration of cisplatin, the elevation of ROS biomarkers in renal tubular and kidney slices in addition to the whole body was observed in the in vitro and in vivo studies on animals. Three theories try to explain the mechanisms behind the induction of oxidative stress by cisplatin. One of them is that the metabolites of cisplatin are highly reactive which gives them a high affinity to bind to thiol groups including GSH which is a well-known antioxidant resulting in a depilation or inactivation of GSH [73]. This mechanism causes an imbalance between ROS and antioxidants which increases the endogenous accumulation of ROS in the cell and that leads to the increase the oxidative stress [74]. The second mechanism relies on disturbing the respiratory chain as a result of the dysfunction of mitochondria due to cisplatin which will increase ROS levels. mitochondrial-targeted supporting involvement of ROS mitochondrial generation in cisplatin-induced renal injury was due to the protective effects of manganese superoxide dismutase (SOD) [75]. Intriguingly, within the same investigation, the introduction of catalase (CAT) into mitochondria did not yield substantial protective outcomes, implying a potential predominance of superoxide over hydrogen peroxide as the principal oxidative species originating from mitochondria. In contrast, recent evidence indicates that CAT and its derivatives not only mitigate cisplatin-induced nephrotoxicity but also enhance the therapeutic efficacy of cisplatin in combating solid tumors [76].

Several antioxidants including NAC, melatonin, selenium and vitamin E have shown renoprotective effects in different animal studies [25]. The effectiveness of using antioxidant substances to reduce kidney damage during cisplatin-based chemotherapy in humans is uncertain. A study with 48 cancer patients found no statistically significant differences in the degree of cisplatin-induced kidney impairment among the groups that received a dietary supplement including vitamin C, vitamin E, and selenium and the group that received a placebo [77]. An earlier study in 1999 showed that taking oral vitamin E can reduce the incidence and extent of cisplatin-induced peripheral neurotoxicity and nephrotoxicity [78].

Factors including adherence by patients to the regimen and the dose of antioxidant supplements are probable drivers of trial outcomes. There is a growing interest in investigating antioxidant agents from sources that are natural in addition to manufactured molecules. Natural antioxidants have the potential to detoxify ROS in the kidneys without affecting the anticancer effectiveness of cisplatin. The bioactive components of these natural compounds have not been revealed, but the confirmation of their kidney-protective properties in human patients could indicate interesting therapeutic possibilities [25].

1.4. The toxic effects of cisplatin

Nephrotoxicity

The nephrotoxic effects of cisplatin have been extensively investigated. However, the exact pathways remain unknown. Histological examination of individuals with cisplatin-induced renal failure has revealed alterations mostly in the collecting duct, distal tubule, and S3 region of the proximal tubule. Nephrotoxicity typically arises from both direct and indirect harm to the kidneys. The intricate mechanism by which cisplatin causes damage to the tubules and consequent renal failure involves multiple biomolecules and interrelated pathways [79].

Originally, it was assumed that cisplatin reached cells through passive diffusion. Recent examination shows that transporter-mediated or better diffusion may also make a contribution to the uptake of cisplatin with the aid of renal tubular cells [80]. The involvement of organic cation transporters (OCTs) and copper transporter 1 (CTR1) in this system has been counseled. Research has verified that cimetidine, that is processed via OCT2, diminishes the absorption of cisplatin and its harmful results on the kidneys, both in live organisms and in laboratory settings. Furthermore, it has been demonstrated that the decrease in CTR1 expression leads to a lower in the absorption and harmful results of cisplatin [79-81].

Cisplatin has the ability to attach itself to GSH molecules within the kidney forming GSH conjugates. This process is facilitated by an enzyme called glutathione-S-transferase [82]. Further enzymatic breakdown of these conjugates such as cysteine-S-conjugateβ-lyase produces highly reactive thiols, which have the potential to bind to essential proteins within proximal tubular cells, worsening nephrotoxicity [82 – 83].

Hydrolyzed cisplatin products have the ability to attach to nuclear DNA, creating connections inside and between DNA strands. This interferes with the processes of replication and transcription, leading to the initiation of apoptosis. The tumor suppressor protein p53 is essential in facilitating cisplatin-induced apoptosis by activating genes that promote cell death [84].

The buildup of cisplatin in renal mitochondria leads to dysfunction, which is characterized by a rise in the formation of ROS, disturbance of mitochondrial energetics, and inhibition of fatty acid oxidation. The limited repair capacities of mitochondrial DNA and proteins make them prime targets for damage caused by cisplatin [85].

The hydrolysis of cisplatin produces rather reactive intermediates that interact with cell oxidative phosphorylation activates the eighth caspase, which includes GSH, resulting in oxidative damage. Mitochondrial breathing complexes also are impacted, which worsens the formation of ROS. The injection of cisplatin results in an elevation of nitric oxide synthase activity, which in turn contributes to the incidence of nitrosative stress [86]. It has been reported that the administration of N-G-nitro-L-arginine methyl ester can decrease the NOS and show a reduction in the nephrotoxic effect of cisplatin in rats [87].

Cisplatin induces an inflammatory response marked by using the extended expression of inflammatory cytokines and chemokines, activation of NF-κB, and migration of immune cells to the kidneys [88]. Tumor necrosis factor α (TNF-α) is vital in coordinating this inflammatory cascade [89].

Also, it has been found that cisplatin can affect the signaling of specific pathways such as Mitogen-Activated Protein Kinase (MPAK) including Jun N-terminal kinase, extracellular signal-regulated kinase and p38. Those pathways are involved in various cellular functions such as cellular distress, inflammation and apoptotic cell death [90,91]. The particular functionality of MPAK seems to be very complicated and not fully understood. Few in vitro and in vivo research described the cellular activation pathways of MPAK in various patterns of activation [25, 92].

The nephrotoxicity of cisplatin is dose-dependent whereas high doses cause nephrotic necrosis while lower doses initiate apoptosis. The apoptotic effect of cisplatin toxicity is dominant to necrosis [93]. Several mechanisms control the pathways of apoptosis which are described as intrinsic and extrinsic pathways. The intrinsic pathway of cisplatin-induced nephrotoxicity involves the mitochondria mainly by triggering cytosolic dependent and independent apoptotic pathways by releasing cytochrome C and endonuclease G from mitochondria [94,95]. Usually, it is followed by the extrinsic pathway which activates the caspase-8 which in return increases Fas expression that leads to apoptosis [94 - 96]. On the other hand, the stress on the endoplasmic reticulum is involved in triggering caspase-12 which is responsible for tubular cell apoptosis. Also, p53 has an important role in cisplatin-induced apoptosis by modulating the gene expression due to the adduct of cisplatin metabolites on DNA in addition to modulating the mitochondrial permeability [94],

Cisplatin can interfere with the regulation of the cell cycle by binding to a protein family called cyclin-dependent kinase (CDK) and upregulates p21 gene which is known as the cell cycle-suppressing gene. Thus, those factors are a key role in tubular toxicity and apoptotic cell death induced by cisplatin [97 – 99].

Hepatotoxicity

Cisplatin does not have a significant hepatotoxic effect. However, an overdose of cisplatin might show a toxic effect. The main mechanism behind the hepatotoxic effect of cisplatin is to trigger oxidative stress [100]. The depletion of GSH which is known as an antioxidant might facilitate hepatotoxicity by decreasing the GSH reductase and increasing the levels of glutathione peroxidase (GSH-Px) during cisplatin therapy [101]. The adverse effect in the liver usually is measured by general biomarkers such as AST and ALT. Also, it has been found that cisplatin is able to enhance the activity of cytochrome enzymes particularly cytochrome-P450-2E1 enzyme which is responsible for liver toxicity [102].

Neurotoxicity

Cisplatin is thought to set off peripheral neuropathy in around 30% of patients receiving cisplatin, with peripheral sensory neuropathy often discovered as a dose-dependent manner [103,104]. This neurotoxicity commonly occurs with high doses of cisplatin, and two important mechanisms have been proposed to explain its incidence: harming DNA and harming mitochondrial DNA. Damage to nuclear DNA can also disrupt crucial mobile methods essential for nerve function, potentially interfering with neuronal signaling pathways and leading to neuropathic signs and symptoms [105]. Similarly, harm to mitochondrial DNA should impair energy production and oxidative strain regulation within nerve cells, exacerbating neuropathic symptoms. Understanding those mechanisms is essential for growing techniques to mitigate cisplatin-precipitated peripheral neuropathy and enhance the quality of life of patients who are undergoing anticancer chemotherapy.

Ototoxicity

Hearing loss or ototoxicity is another adverse effect that is observed in patients who undergo with cisplatin chemotherapy regime [1]. The main mechanism behind ototoxicity is triggering ROS in cochlea cells by the administration of cisplatin which results a hearing loss in patients [106]. However, children are more susceptible to experience this adverse effect compared to adults [106]. This adverse effect is very challenging, especially with cancer-free patients who have been cured from cancer, but they still have to suffer from the consequences of cisplatin therapy.

Cardiotoxicity and Hematological toxicity

Hematological and cardiotoxicity are rare adverse effect associated with cisplatin. However, in the recent 20 years, the reports of cisplatin-induced cardiotoxicity have been increased. The role of oxidative stress is very important in both of those toxicities [1]. Hypomagnesemia is due to cisplatin treatment is responsible of acute vascular events [107]. Increase the inflammatory response and mediators which are triggered by ROS generation might cause damage in cardiomyocytes [107].

1.5. Clinical management of cisplatin-induced nephrotoxicity

The clinical management of cisplatin-induced nephrotoxicity is limited to supportive treatment to support kidney function. Administration of isotonic solution via IV-line aiming to maintain hydration and urine flow in order to minimize kidney damage. On the other hand, magnesium depletion due to cisplatin therapy must be replaced by magnesium supplementation to decrease the toxic effect of cisplatin on the kidney [108]. The administration of osmotic diuretic such as mannitol does not show good evidence of protecting the kidney. However, it is used as routinely as prophylactic nephroprotective regime [108].

1.6. Nephroprotective strategies against cisplatin-induced nephrotoxicity

Currently, there are multiple ways have studied in order to decrease the nephrotoxicity which is induced by cisplatin, various mechanisms and agents are involved for this aim having the same strategic aim with different techniques. [25] One of the main strategies is to decrease oxidative stress either by decreasing ROS or providing exogenous anti-oxidants. One of the most famous antioxidants is NAC which works as a precursor of

GSH [25]. The anti-oxidant and the nephroprotective effect of NAC were investigated in many in vitro, in vivo and even in clinical studies [109]. Several dietary supplements were studied in the same manner as NAC such as vitamin C, vitamin E, capsaicin and gum arabica [110 – 113]. Despite, the famous antioxidants such as NAC, many natural products have been investigated to evaluate their activity against cisplatin-induced nephrotoxicity.

Minimizing the nephron uptake of cisplatin by inhibiting the OCTs by cimetidine shows an excellent nephroprotective effect in several in vitro and in vivo studies [114 – 115]. However, it might interfere with the anti-cancer activity of cisplatin in kidney tumors [116].

Another mechanism in decreasing the toxicity of cisplatin in the kidney is by inhibiting the enzymes that are responsible for metabolizing or bioactivating of cisplatin such as γ-glutamyl transpeptidase and cysteine-S-conjugate b-lyase [117]. Acivicin is a glutamine analog that has an inhibitory effect on γ-glutamyl transpeptidase in vitro and in vivo [118].

Roscovitine, an experimental drug, has activity against type 2 cyclin-dependent kinase which initiates tubular apoptosis [25]. On the other hand, Amifostine is an FDA-approved cytoprotective agent that can provide nephron and neuroprotective activity against cisplatin-induced toxicity without antagonizing the anti-cancer activity of cisplatin [119].

Inflammation plays an important role in elevating the toxic effect of cisplatin by causing kidney injury [25]. The use of TNF-α inhibitor or salicylate might decrease the nephrotoxicity of cisplatin [120, 121]. The interference of inhibiting the inflammatory mediators and the anti-tumor activity of cisplatin is unclear and needs further investigation.

1.7. N-ACETYLCYSTEINE

1.7.1. Historical overview of N-acetylcysteine

NAC serves as a synthetic derivative of the naturally occurring amino acid L-cysteine which acts as a precursor of cysteine [122]. The chemical structure of NAC includes a thiol functional group which facilitates its permeability through the membranes of cells.

This functionality is enabling to reinforce the intracellular reservoirs of cysteine that is critical for the biosynthesis of GSH. GSH works as an important antioxidant molecule that performs an imperative position in the cellular protection mechanism against oxidative stress and ROS. In other words, the significance of NAC comes from its ability to preserve the cellular homeostasis in human and animal cells. Eventually, this system will elevate the levels of GSH and intensify the detoxification capacity in opposition to hydrogen peroxide (H_2O_2) via augmenting the interest of antioxidant enzymes including of GSH-Px and thioredoxin [123].

The chemical structure of NAC contains sulfhydryl functional group (-SH) combined with an acetyl group ($-COCH_3$) attached to the amino group (NH_2). This configuration allows thiols (RSH) within NAC to serve in a manner as an electron donor that rendering it susceptible to oxidation by radicals. The antioxidant properties of this substance come from its ability to interact with different reactive oxygen and nitrogen species. For example, hydroxyl radical (OH·), nitrogen dioxide (NO_2·), carbon trioxide ion (CO_3•), and nitroxyl (HNO·). In addition, NAC reacts in a slower manner with some free radicals (e.g. as superoxide radical anion (O_2•), hydrogen peroxide (H_2O_2•), and peroxynitrite (ONOO·). These reactions play a vital role in reducing oxidative stress within cells [123].

1.7.2. NAC as a pharmacological agent

When it comes to the therapy of paracetamol overdose toxicity, the first-line medication that is recommended is NAC Appropriate dosing of APAP that might possibly be hazardous has received clearance from the FDA [124]. As an antidote, APAP is almost completely effective if it is delivered within eight hours of the consumption of the substance [125]. The FDA approval was given for the management of different illnesses including diseases that are characterized by aberrant mucous discharges, such as pneumonia, bronchitis, cystic fibrosis, individuals who have had tracheostomy, and a variety of postoperative pulmonary problems. As an additional benefit, it might be utilized prior to diagnostic bronchoscopy in order to reduce mucus clogging [126]. Besides the various clinical applications of NAC, it has also many off-label indications, such as the management of acute hepatic failure, the prevention of contrast-induced nephropathy, and the treatment of keratoconjunctivitis sicca by topical application

[127]. Through the elucidation of NAC's indications, mechanism of action, delivery protocols, side effects, contraindications, monitoring measures, and toxicity, this article equips healthcare personnel with the ability to optimize patient care outcomes in situations when NAC is advantageous. The objectives include elucidating the mechanism by which NAC is responsible for acetaminophen toxicity and conditions that require mucolytic activity, reviewing its indications, providing specifics regarding its administration in cases of APAP toxicity and lung conditions that require mucolytic therapy, and highlighting the pivotal role that interprofessional care coordination plays in improving patient care delivery for those who are benefiting from NAC therapy [124].

Beyond its approved uses, NAC is also employed off-label for acute hepatic failure, preventing contrast-induced nephropathy, and topically treating keratoconjunctivitis sicca [127]. Research explores its efficacy in countering xenobiotics featuring free radical or reactive metabolite toxicity. Studies indicate its effectiveness in acute exposures to cyclopeptide-containing mushrooms and carbon tetrachloride. Additionally, animal and human tissue studies suggest its potential in mitigating cisplatin-induced nephrotoxicity [128], although clinical evidence remains limited. NAC's therapeutic potential extends to addressing chronic valproate hepatotoxicity and acute hepatotoxicity induced by pennyroyal or clove oil ingestion [129 - 131].

Exploratory investigations into NAC's applications include its role as an antineoplastic agent and its potential in managing psychiatric disorders such as schizophrenia, bipolar disorder, and depression [132]. Moreover, it shows promise in gastrointestinal conditions like hepatorenal syndrome [133], Helicobacter pylori infections, and necrotizing enterocolitis [134]. In critical care settings, NAC may benefit patients with lung injury, cardiac injury, multiorgan dysfunction, and sepsis, while also showing potential in addressing haematological conditions like sickle cell disease. Case reports accidentally NAC administration shows a positive impact on neurological status in comatose patients suffering from carbon monoxide poisoning [135].

When a patient has taken an excessive amount of acetaminophen (APAP), the delivery of NAC is contingent upon determining the patient's potential for hepatotoxicity. As part of this assessment, a detailed history will be gathered, a physical examination will be performed, and serum APAP and transaminase levels will be measured [136].

It is essential to have a complete history, which should include information on the quantity of APAP that was ingested as well as whether it was consumed all at once or over a period of time. In addition, it is vital to be aware of any other compounds that are being taken at the same time, such as opioids or anticholinergic medications, because these substances have the potential to influence the absorption of APAP. Additionally, it is important to take into consideration factors such as malnutrition, alcoholism, or cirrhosis, all of which are associated with decreased GSH stores. As the extended-release formulation of APAP might result in delayed peak serum concentrations, it is essential to ascertain whether the APAP that was taken was of the usual formulation or the extended-release kind. As a further point of interest, it is essential to evaluate the utilisation of medications that stimulate CYP2E1, such as isoniazid or prolonged alcohol intake, because these substances increase the likelihood of hepatotoxicity [137].

Following an acute single intake of APAP, the Rumack-Mathew Nomogram is a useful tool for establishing whether or not it is necessary to begin the administration of NAC and for assessing the potential for hepatotoxicity. For ingestions that take place within a time frame of less than four hours, the nomogram is shown with the serum APAP levels at four hours. If it is above the treatment line, it is suggested to begin NAC therapy; if it is below the treatment line, the risk of hepatotoxicity is negligible [138].

It is preferable to wait for the serum APAP levels to be obtained within eight hours before making a decision on the commencement of NAC treatment for ingestions that occur between four and twenty-four hours after the ingestion. NAC can be begun empirically and stopped if the levels fall below the treatment line if the APAP levels are not known until more than eight hours after the administration of the medication.

In the event that the dose that was consumed was unclear or if the consumption lasted for more than twenty-four hours, the initial dose of NAC should be administered, and then the APAP and transaminase levels should be determined. It is possible to continue administering NAC even if the levels of APAP are higher than 10 mg/L or if transaminases are raised [139 - 140].

It is recommended that NAC treatment be initiated in cases of chronic intake if the levels of APAP are more than 20 mg/L or if transaminases reveal an increase. It has been shown that there are no recognized fetal concerns connected with the commencement of NAC treatment for pregnant women, and the dose procedures are comparable to those used for the general population [140].

It is possible to deliver NAC either orally or intravenously, and the efficiency of either method is quite similar. The 21-hour intravenous protocol and the 72-hour oral dosage protocol are two examples of regimens that are often utilised. The administration of NAC is suggested for individuals who are at risk of experiencing hepatotoxicity, and it should be maintained in the event that hepatotoxicity occurs. The discontinuation of treatment takes place either after the regimen is finished or when the hepatotoxicity situation is resolved, whichever comes first. Both methods are equally effective in avoiding and treating APAP toxicity; however, intravenous administration is favoured in cases where hepatic failure has already been established or when oral NAC cannot be tolerated owing to severe nausea or vomiting.

NAC is available for intravenous usage in vials containing 30 milliliters of a 20% concentration; however, it must be diluted before being administered. Both 10% and 20% oral NAC are available in vials of 10 milliliters each, and both require dilution [140].

An initial loading dosage is included in the dosing plan for the 21-hour intravenous regimen, which is then followed by maintenance doses. Oral NAC is administered in a 72-hour regimen, which begins with a loading dosage and continues with successive doses every four hours [139.

It is recommended that the administration of NAC be continued until the levels of APAP become undetectable, the PT/INR gets closer to normal, the encephalopathy goes away, and the transaminase levels either normalize or trend lower with the AST being below 1000 U/L. There are predetermined periods at which follow-up testing takes place, and if it is deemed essential, the patient's NAC treatment may be continued until either their health improves or they obtain a liver transplant [140].

1.7.3. The Clinical Applications of NAC

Numerous diseases are connected to cellular oxidative stress [141 – 145]. Cells have numerous defensive mechanisms against oxidative stress [146]. Nearly all cells synthesise and maintain high amounts of GSH which is a vital defensive endogenous biomolecular substance which neutralizes ROS [143].

The precursor of GSH is NAC, which is a synthetic derivative of L-cysteine. In addition to their effects on oxidative stress, NAC also has an influence on mitochondrial dysfunction, apoptosis, and inflammation. It indirectly affects glutamate and dopamine [147].

The therapeutic potential of exogenous antioxidants like cysteine and GSH has long been studied. Clinical usage of NAC dates back over 50 years to its in vitro and in vivo therapeutic studies [148]. Between the 1960s to 1970s, the study of NAC was moved to another level [149]. Initially, its impact was investigated with the patients who suffer from cystic fibrosis due to its extraordinary mucolytic activity followed by the establishment of NAC as an antidote for paracetamol toxicity in the 1970s. However, the research about NAC did not stop here, since 1980 scientists have been trying to understand and study the impact of NAC in several diseases that have an etiological oxidative stress origin [150, 151].

NAC and Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is a common and preventable condition characterized by a combination of obstructive bronchiolitis (small airways disease) and emphysema (deterioration of lung tissue). The extent to which each component affects individuals can vary [152]. The continuous oxidative stress, caused by both internal and external oxidants, such as inhaled cigarette smoke and other pollutants, triggers the long-lasting inflammation that characterizes COPD [144, 152, 153]. The levels of GSH outside and within the cells in individuals with chronic obstructive pulmonary disease (COPD) are frequently abnormal. This might potentially speed up the course of the disease since the body is unable to maintain normal GSH levels [154].

While lung function typically declines with age, this reduction is markedly accelerated in COPD sufferers, primarily due to the cumulative impact of prolonged oxidative stress. Consequently, COPD can be conceptualized as a syndrome of expedited pulmonary aging, characterized by diminished elasticity and respiratory impairment beginning as early as age 30. This notion posits that early intervention to counter oxidative stress in younger individuals could potentially forestall disease advancement.

Despite COPD’s unpredictable trajectory towards severe health deterioration, which usually progresses slowly, there is a window for preventive strategies, such as NAC, to be efficacious [155].

The proposition that NAC therapy might thwart COPD exacerbations, characterized by periods of symptomatic decline often associated with heightened pulmonary inflammation, was investigated in the BRONCUS, HIACE, and PANTHEON trials, which yielded variable results due to differences in dosage and target populations [156 - 158]. In the BRONCUS trial, a randomized and placebo-controlled study, the oral administration of NAC at a dosage of 600 mg per day for a duration of three years did not have any effect on the pace of fall in forced expiratory volume in one second (FEV1) or the yearly frequency of exacerbations [156]. This result was especially apparent in patients who were receiving inhaled corticosteroids (ICS), maybe because of the relatively modest amount of NAC that was administered.

Following that, further trials investigated a greater dosage of NAC, which was 1200 mg per day (600 mg twice day). In comparison to the placebo, high-dose NAC was shown to considerably enhance lung function and reduce the frequency of exacerbations, as indicated by the findings of the HIACE trial [157]. Over the course of a year, this study included Chinese patients with steady COPD. While high-dose NAC treatment cut down on the number of exacerbations patients had by a large amount, it also made it take longer for patients to have their first exacerbation. This was determined through a post-hoc analysis that was done on the HIACE research. A patient who was at a minimal risk of suffering exacerbations, on the other hand, did not experience this phenomenon [159]. Similarly, the comprehensive PANTHEON experiment with Chinese patients found a 22% reduction in acute exacerbations of COPD (AECOPD) after a year of high-dose NAC [158]. This research was randomized, double-blind, and controlled with a placebo [158]. The efficacy of NAC emerged from the sixth month onwards, suggesting a delayed but sustained preventive effect with regular treatment.

Post-hoc analysis of the PANTHEON study supported the finding that NAC diminishes the rate of COPD exacerbations, particularly among patients with a smoking history or those not receiving ICS treatment [160]. Therefore, NAC might serve as an alternative to ICS-containing therapies in these subpopulations. The meta-analysis of NAC's effectiveness in avoiding COPD exacerbations found varying responses to low (≤ 600 mg/day) and high (> 600 mg/day) dosages [161]. 13 trials with 4,155 patients found that NAC treatment significantly reduced COPD or chronic bronchitis exacerbations (relative risk 0.75, 95% CI 0.89–0.97, $p < 0.01$). NAC was more useful in individuals without airway obstruction, but high-dose NAC still helped. NAC at dosages > 1200 mg/day may prevent exacerbations in COPD patients with chronic bronchitis and airway obstruction, but conventional doses (600 mg/day) may sufficient in the absence of airway obstruction [161].

The 2020 Global Initiative for chronic obstructive pulmonary disease (GOLD report includes NAC as an adjunctive therapy for COPD management, along with other thiol-based medications, due to recent trials showing its ability to reduce COPD exacerbations, even in ICS-treated patients [152].

NAC and Idiopathic Pulmonary Fibrosis (IPF)

IPF is a chronic, fibroproliferative interstitial pneumonia of unclear aetiology that mostly affects elderly persons and causes lung function to decrease and has a bad prognosis. Oxygen radicals and GSH deficiency affect IPF development and progression [162]. Thus, NAC and other thiol-based medicines may help treat IPF [163]. Several preclinical models show that NAC inhibits profibrotic pathways. Lung fibroblasts respond to GSH in vitro [164]. Oral NAC raises lung GSH levels in bronchoalveolar lavage fluid in IPF patients, improving lung function.

High-dose oral NAC (600 mg, three times daily) was tested in 182 IPF patients for a year with prednisone and azathioprine in the randomized, placebo-controlled IFIGENIA study [165]. NAC retained lung function (vital capacity and single-breath carbon monoxide diffusing capacity) better than conventional treatment with placebo without substantial safety differences. Later, well-designed, randomized, placebo-controlled studies with NAC 600 mg three times a day, either alone or in combination with prednisone and azathioprine [166], failed to show benefit in IPF treatment and highlighted safety concerns [167]. An investigation of single-nucleotide polymorphisms (SNPs) in the lung host defence genes TOLLIP and MUC5B revealed that the IPF patient's genotype, especially the TOLLIP rs3750920 genotype, may affect NAC's efficacy. The PANTHER-IPF experiment examined the safety of pairing prednisone and azathioprine with NAC. This SNP analysis used trial data [167].

The recent phase 2, placebo-controlled PANORAMA study examined the safety and tolerability of NAC plus pirfenidone in 123 IPF patients [168]. This study examined exploratory effectiveness metrics such forced vital capacity, carbon monoxide diffusing capacity, and six-minute walk distance. Though the trial was insufficient to detect a meaningful difference, adding NAC to pirfenidone did not improve its safety or tolerability or forced vital capacity.

NAC's therapeutic promise in IPF is debated, especially in some patient subgroups. Future research on bearers of the TOLLIP rs3750920 genotype may confirm NAC's advantages for this subgroup before concluding its IPF therapeutic potential [169]. A multicenter clinical study targeting this subpopulation is underway.

NAC in Obsessive-compulsive Disorder and Autism

The potential therapeutic benefits of NAC in the treatment of autism spectrum disorder (ASD) and obsessive-compulsive disorder (OCD) have garnered considerable interest [122]. This substance's ability to control neurotransmitters like glutamate and dopamine, as well as its antioxidative properties, have prompted research into its potential use in OCD. Compared to a placebo, NAC alleviated OCD symptoms after 12 weeks of therapy, according to research [170]. The Clinical Global Impression Scale and the Yale-Brown Obsessive-Compulsive Scale were used to evaluate the improvements. The administration of NAC took place. Nevertheless, the effectiveness of NAC in treating obsessive-compulsive disorder (OCD) remains uncertain because systematic studies have only found a limited amount of compelling data to support its efficacy [171].

In the domain of ASD, examinations into the role of NAC are ongoing. Although certain studies imply that oxidative stress markers may be elevated in children with ASD, suggesting a potential utility for NAC in addressing irritability, the substantiation is inconsistent [173]. Diverse studies have delineated alterations in levels of GSH, GSH-Px, cysteine, methionine, and glutathione disulfide (GSSG) in children with ASD compared to controls. Nevertheless, findings pertaining to other markers such as homocysteine, SOD, and cystathionine have manifested inconsistencies [173].

Preliminary models have intimated a conceivable function for NAC in bolstering mitochondrial metabolism in ASD [174]. Nevertheless, the outcomes of clinical studies probing the repercussions of NAC on fundamental symptoms of ASD have been varied. Extensive clinical trials are indispensable for a more profound comprehension of the potential benefits of NAC therapy for ASD [122].

NAC as an Antidote for Paracetamol Overdose

Acetaminophen or paracetamol is considered a very safe drug with a wide therapeutic index. It does not cause toxicity under normal circumstances as long as it is administrated in the therapeutical dose range. The reason behind this safe index is that most of the administrated dose is metabolized by phase II reaction which is called conjugation reaction, and a very low concentration of the drug undergoes CYP450 2E1 metabolism which is responsible for the active toxic metabolite which is "N-acetyl-p-benzoquinone imine" or "NAPQI" [137]. The toxicity occurs when a large dose of paracetamol is administered and cannot be efficiently metabolized by conjugation reaction due to the depletion of hepatic GSH so NAPQI can form a protein adduct leading to liver damage and this also includes the mitochondrial proteins [174]. Those types of adducts have a leading role in initiating oxidative stress. This mitochondrial protein adducts are pivotal as they instigate initial oxidative stress, which is subsequently magnified through a mitogen-activated protein (MAP) kinase cascade, culminating in the activation of c-jun N-terminal kinase (JNK) [174].

The translocation of phospho-JNK to mitochondria results in increased electron leakage from the electron transport chain and the generation of superoxide. This superoxide reacts with nitric oxide to form peroxynitrite which is a highly reactive oxidant within the mitochondria [174]. The ultimate oxidant thought to be in charge of opening the mitochondrial membrane permeability transition pore is peroxynitrite which leads to hepatic cellular death [175]. The activation of MPTP is able to halt ATP production and induces matrix swelling which leads to the breaking down of the outer mitochondrial membrane and the resulting release of intermembrane proteins that includes endonuclease G and apoptosis-inducing factor [176]. These proteins translocate to the nucleus, causing DNA fragmentation and initiating cellular necrosis [176]. These mechanisms, observed in murine models and human hepatocytes, apply to acetaminophen-induced cell death in overdose patients [177].

Initially, antidotes capable of replenishing hepatic GSH levels were explored based on the early understanding of paracetamol liver-toxicity which included reactive metabolite formation and GSH depletion [58]. There are several antidotes that demonstrated efficacy in animal models and patients [151]. However, NAC quickly became the preferred antidote due to its superior efficacy and minimal adverse effects [151]. NAC is most effective when administered within the first 8-10 hours post-overdose but still confers benefits if given later [124].

Among patients with plasma acetaminophen concentrations indicating a risk for hepatotoxicity, only 6-7% developed liver injury when treated with NAC within 10 hours of overdose, while 26-29% exhibited hepatotoxicity if treated between 10-16 hours, and 40-60% sustained liver injury if NAC was administered between 16-24 hours [178, 179]. These outcomes were significantly better than historical controls. A nomogram was developed to guide NAC treatment by plotting acetaminophen plasma concentrations against the time since overdose [180]. Although the current nomogram is conservative and reliable, it may not fully assess risk from a single measurement, as patients initially below the treatment line can surpass the threshold post-overdose [181].

Early NAC treatment in animal studies showed protection by scavenging NAPQI and preventing protein adduct formation [182]. While NAC does not directly react with NAPQI, it supplies cysteine for GSH synthesis, which then reacts with NAPQI [183]. GSH also scavenges peroxynitrite and acts as a co-factor for hydrogen peroxide detoxification by GSH-Px [175]. Excess NAC not used for GSH synthesis converts to Krebs cycle intermediates, supporting mitochondrial bioenergetics and increasing ATP levels in hepatocytes [184]. NAC also protects against mitochondrial dysfunction, a key factor in acetaminophen-induced liver injury [175]. Thus, NAC’s multiple modes of action help explain its benefits, even when administered significantly post-overdose [134].

Recovery from acetaminophen-induced liver injury depends on the regeneration and replacement of necrotic hepatocytes, which relies on mitochondrial biogenesis [185]. While early NAC treatment limits injury, progressive administration of NAC might the capability of liver regeneration by impairing mitochondrial biogenesis, an adaptive response crucial for maintaining or restoring homeostasis following mitochondrial dysfunction [185]. This is a notable concern with prolonged NAC treatment.

Despite the widespread use of NAC, no randomized clinical trials have been conducted to establish its efficacy and optimal dosage for ethical reasons [187]. Early IV treatment of overdose patients with an empirical NAC dose showed complete protection for those treated within 10 hours [151]. Follow-up studies indicated that early NAC treatment resulted in significantly fewer cases of severe liver injury compared to historical controls [124]. In the US, oral NAC treatment showed similar efficacy, though its effectiveness declined when treatment commenced later [178, 178]. The high efficacy of early NAC treatment and its minimal adverse effects compared to other hepatoprotective agents established NAC as the antidote of choice for paracetamol toxicity [124]. Late-presenting patients with paracetamol-induced fulminant liver failure also had survival benefits with NAC therapy [139].

Due to the absence of randomized clinical trials, there is no globally accepted protocol for NAC usage in the management of paracetamol toxicity. Initially, IV protocols were utilized in the UK, while a 72-hour oral protocol was developed in the US due to regulatory constraints [187]. In 2004, an IV preparation was approved in the US. Retrospective comparisons indicated slightly better outcomes with early IV treatment, no difference between 12-18 hours, and lower risk with the 72-hour oral regimen [189]. A more recent study compared the administration time of NAC found no significant variation in the endpoints when it is administrated within 8 hours of the intoxication [190]. Additionally, an abbreviated 12-hour IV NAC dosing regimen proved as effective as the 20-hour regimen with fewer adverse effects [191].

1.7.4. NAC protective effect against cisplatin-induced toxicity

Cisplatin is one of the most effective anticancer drugs. However, the clinical use of this drug might be limited sometimes due to its nephrotoxic effects on the kidneys and liver. Multiple studies have investigated the potential effect nephroprotective effect of NAC due to its antioxidative activity and anti-inflammatory effect to minimize the nephrotoxic impact of cisplatin [192 – 197].

A rat model experiment tried to investigate the effect of NAC on cisplatin-induced nephrotoxicity. The results showed that NAC is capable to enhance the activity of oxidative stress parameters such as SOD, GSH-Px, and CAT. In addition to decreasing inflammation, the study found a better histological architecture of the group of rats that were injected with cisplatin and NAC compared to cisplatin alone. Moreover, this investigation has found a synergistic nephroprotective between NAC and taurine against cisplatin toxicity [192].

Another research has studied the synergistic effect of NAC and chlorogenic acid on cisplatin-induced nephrotoxicity. The study found that NAC has declined tubular necrosis on the histological level. On the molecular level, the research found that NAC has decreased inflammation and LPO by enhancing the antioxidant defence system [193]. Similar studies were conducted for the same aim. They used various drugs, supplements and natural products such as nebivolol, taurine and vitamin E along with NAC to evaluate the synergistic nephroprotective effect [194 – 197].

1.8. WHEY PROTEIN

1.8.1. Overview of Whey Protein

Milk protein consists of casein protein, which represents approximately 80% of milk protein; and whey protein which represents the remaining fraction of milk protein which is almost 20%. Milk protein has a high nutritional value due to its functional advantages and remarkable antioxidant properties [198]. Whey protein, which is also known as milk plasma, contains multiple fractions such as "β-lactoglobulin, α-lactalbumin, immunoglobulins, lactoferrin, lactoperoxidase, and glycomacropeptide" which are biologically active constitutes [198]. That is why WP is considered an outstanding source of amino acids which in turn have significant contributions in the antioxidant properties of WP [199].

The hydrolysates of WP include a significant quantity of sulfur-containing amino acids, which contribute to the antioxidant properties of the hydrolysates. It has been proven that WP possesses antioxidant properties by a variety of in vitro tests, including SOD anion radical scavenging activity assay, the hydroxyl radical scavenging activity assay, and the reducing power assay [200]. On the other hand, there are not many research that have been conducted on the effects of WP-derived antioxidative peptides on live cells. Furthermore, it is not yet known if these peptides have any protective benefits against oxidative cytotoxicity in neuronal models [201].

There is no previous research had investigated the effect of WP against cisplatin-induced nephrotoxicity. Thus, this study aims to investigate the protective effect of WP on liver and kidney against cisplatin-induced toxicity.

1.8.2. Anti-oxidant properties of Whey Protein

The role that WP performs as an antioxidant in vivo is extremely important. Increased activity of major antioxidant enzymes, including as SOD, CAT and GSH-Px, has been demonstrated to regulate the redox state of endothelial cells and moderate oxidative stress. This is accomplished by the enhancement of the activity of these enzymes. While SOD is responsible for catalyzing the conversion of superoxide radicals to hydrogen peroxide, CAT and GSH-Px are responsible for further detoxifying hydrogen peroxide into water and oxygen, therefore protecting cells from oxidative damage. In studies conducted on animals, it has been found that administration of WP has been correlated with improvements in a variety of biomarkers of oxidative damage. These biomarkers include protein carbonyls and TBARS which all demonstrate the versatile and advantageous function that WP plays [202].

1.8.3. Therapeutic Applications of Whey Protein

Whey protein concentrates have been studied extensively for cancer prevention and treatment, primarily due to their ability to stimulate GSH, which boosts immunity and detoxifies potential carcinogens. Research indicates that whey can enhance GSH levels in tissues, improve immune responses [203], and reduce oxidative damage through its iron-binding capacity [204].

Animal studies have shown that whey and its components, such as lactoferrin and beta-lactoglobulin, can significantly lower tumor incidence and inhibit cancer cell growth [205, 206]. Additionally, bovine serum albumin in whey has demonstrated growth inhibition in human breast cancer cells [207]. Whey has also been effective in preventing and treating chemotherapy-induced oral mucositis in animal models by reducing basal epithelial cell proliferation [208].

Clinical trials on whey protein and cancer have been limited. High GSH concentrations in tumor cells might confer resistance to chemotherapeutic agents. A small 1995 trial involving patients with various metastatic cancers showed mixed results with whey protein concentrate over six months. Some patients showed tumor regression or stabilization, while others experienced disease progression. These mixed outcomes highlight the need for larger trials [209].

A recent clinical trial involved 20 patients with stage IV cancers receiving a combination therapy, including 40 g/day nondenatured whey protein concentrate, along with several other supplements. After six months, 16 survivors had improved immune function, higher hemoglobin and hematocrit levels, and reported an improved quality of life. However, no comparison was made between this combination therapy and whey protein alone [210].

In individuals who are afflicted with either Hepatitis B or Hepatitis C, the effects of taking a supplement containing whey protein have been reported to differ. In the beginning, investigations conducted in vitro indicated that bovine lactoferrin has the ability to inhibit infection with the Hepatitis C virus (HCV) in human hepatocyte lines. Because of these findings [211], new clinical studies were conducted in order to investigate this possibility further.

A pilot research was carried out with eleven individuals who were all suffering from chronic HCV. Either 1.8 g or 3.6 g of bovine lactoferrin were administered to each patient on a daily basis for a period of eight weeks. The levels of HCV RNA and serum alanine transaminase were found to be lower in patients who had low viral loads prior to therapy. The individuals who had greater viral loads, on the other hand, did not observe any significant alterations [212].

A more comprehensive dose-response experiment was conducted with 45 people who were infected with HCV. An amount of lactoferrin equal to 1.8 grammes, 3.6 g, or 7.2 g was given to each participant on a daily basis for a period of eight weeks. There were only four patients who presented with signs of a virological response, although HCV RNA was still detected in all of them. After eight weeks of therapy, eight patients showed a reduction in HCV RNA that was at least 50% lower than before. This study emphasized on conducting more research in order to to discover the best dose, duration, and possible advantages of combining WP with traditional therapies such as interferon therapy [213].

Twenty-five individuals who had been diagnosed with either Hepatitis B or C participated in an open trial. For 3 months, participants were administered a total of 12 g of WP twice daily. Patients were given 12 g of casein protein on a daily basis for a period of two weeks prior to beginning therapy with WP. Following the completion of the whey treatment, patients continued to receive casein for an additional four weeks [214].

Among the subset of seventeen patients who were diagnosed with HCV, there were no discernible alterations found. On the other hand, the group that was infected with the Hepatitis B virus (HBV) showed a decrease in the levels of serum lipid peroxidase, in addition to an increase in the activity of IL-2 and NK cells. There were 6 out of 8 patients had decreased levels of serum alanine transferase, whereas five of them had elevated levels of plasma GSH. In light of these findings, it appears that taking a supplement containing WP might be advantageous in the treatment of HBV [214].

The following table contains a summary of some clinical trials that investigate the therapeutic effect of WP.

Table 1 Summary of Clinical Trials of Whey Protein

Medical situation	Dose	Study Length	Key Results
Cancer	30 g per day	24 weeks	Tumour shrinkage in 40% of the patients; ↑ GSH in healthy cells ↓ GSH in cancer cells.
	40 g per day	24 weeks	80% survival; better immune function, more glutathione, and improved quality of life.
Hepatitis B	12 g per day	12 weeks	↓ LPO Improved immune response Improvement in liver enzyme test ↑ GSH
HIV	45 g per day	2 weeks 24 weeks	↑ GSH
Risk factors of CVD	A combination of milk and WP	8 weeks	Improved lipid profile ↓ Systolic blood pressure

2. MATERIALS AND METHOD

2.1. Materials:

1,1,3,3-tetramethoxypropane	Sigma Aldrich
2-Thiobarbituric acid (in 0.05 N NaOH)	Sigma Aldrich
Acetic acid sol. (pH 3.5)	Sigma Aldrich
Analytical and precision balances	
Bovine serum albumin (BSA)	Sigma Aldrich
Bullet Blender homogenizer	Heidolph, Silent Crusher S
Centrifuge	Evolution 300 Centrifuge / Sigma 3-16 PK
Dissection Pans	
Dissection Pins	
Dissection Tray	
Distiled Water	
Ethylenedinitrilotetraacetic acid disodium salt dihydrate (Na2EDTA)	Sigma Aldrich
Eppendorf tube	
Forceps	
Freezer	Arcelik
Glutathione reductase	Sigma Aldrich
GSH	Sigma Aldrich
H_2O_2 solution	Sigma Aldrich
hematoxylin and eosin stains	Sigma Aldrich
Hydrogen peroxide in NaOH	Sigma Aldrich
Incubator	
Micropipettes	
Microscope	
Microscope slides	
NADPH	Sigma Aldrich
Petri dishes	
pH meter	
Phosphate buffer	Sigma Aldrich
Pipette	
Polypropylene tubes	

potassium chloride (KCl)	Sigma Aldrich
Refrigerator	Arcelik
Reusable Oral Gavage Needles	
Scalpel	
Scissors	
SDS	Sigma Aldrich
Sodium azide	Sigma Aldrich
Sodium dodecyl sulfate	Sigma Aldrich
sodium dodecyl sulfate solution	Sigma Aldrich
Spectrophotometer	Thermo Scientific
Stock solution of SOD	Sigma Aldrich
Syringes	
TCA	Sigma Aldrich
tert-Butyl hydroperoxide	Sigma Aldrich
thiobarbituric acid	Sigma Aldrich
Trizma base 50 mM, pH 7.6	
Ultrasonic bath	
Volumetric flasks	
Vortex	
Water bath	

2.2. Method:

2.2.1. Animals Experimental Protocol:

Seven-week-old female Sprague Dawley rats were obtained from Yeditepe University Medical School Experimental Research Center (YÜDETAM) at Yeditepe University (Istanbul, Turkey). The animals were maintained at a controlled temperature of 25 ± 1°C with a 12–12 h light-dark cycle (light cycle, 07:00–19:00). The use of these animals and the procedures performed were approved by the Ethical Committee of Yeditepe University between 31.05.2023 – 06.06.2023 under protocol number 2023-23.

2.2.2. Experimental Design:

35 rats were randomly divided into 5 groups (n = 7). The control group, cisplatin (Cisplatin), cisplatin and NAC (Cis-NAC), cisplatin and whey protein concentrate (Cis-WP); and cisplatin, NAC and whey protein (Cis-NAC-WP). The rats in the control group were orally administered with normal saline (3 ml/kg) daily from day 1 – 6. All the groups except the control were treated with a single injection of cisplatin 7 mg/kg I.P on the second day. For the groups (Cis-NAC, Cis-WP, and Cis-NAC-WP) were administrated 300 mg/kg NAC or whey protein P.O daily as it is shown above. The experimental protocol of the animals is summarized in Table 2.

Table 2 Summary of the experimental animal protocol

Group	Intervention	Dose	Route of Administration
Control	Normal Saline	3 mg/kg	Oral gavage
Cisplatin	Cisplatin	7 mg/kg	I.P
Cis-WP	Cisplatin	7 mg/kg	I.P
	Whey protein	400 mg/kg	Oral gavage
Cis-NAC	Cisplatin	7 mg/kg	I.P
	NAC	300 mg/kg	Oral gavage
Cis-NAC-WP	Cisplatin	7 mg/kg	I.P
	Whey protein	400 mg/kg	Oral gavage
	NAC	300 mg/kg	Oral gavage

2.2.3. Blood collection and biochemistry analysis:

Blood samples were collected intracardially from all groups. The collected blood was left to clot at room temperature and centrifuged at 3,000 rpm for 15 min at 4°C to separate the serum, which was stored at -20°C for biochemical analysis. For biochemical parameters, serum urea and creatinine were determined via a semi-automatic biochemistry analyzer using available commercial kits for each parameter in order to evaluate the nephroprotective effect.

2.2.4. Kidney, liver and body weight ratio:

The body weight of each rat was recorded on the first and the last days of the experimental period for calculation of change in body weight. Moreover, kidneys and livers were excised and weighed immediately for calculation of kidney and liver body weight ratio using the following formula: organ ratio (%) = organ weight (g) ×100/ body weight (g).

2.2.5. Preparation of tissue homogenates and protein assay:

The kidney and liver tissue samples, each weighing 1 gram, were individually obtained from rats. Subsequently, the samples were homogenized using a Bullet Blender homogenizer at 75 x 1000 rpm with the addition of a 1.15% KCl solution surrounded by a separate ice-containing baker in order to maintain cold temperatures. Post-homogenization, the samples underwent centrifugation at 4500 g for 10 minutes at a temperature of +4° C then the supernatants were collected and promptly stored in Eppendorf tubes in a –80° C freezer until required for the protein assay. The Lowery method was employed to ascertain the protein concentration in the supernatants as it has been described by (Aydin et al, 2001)

2.2.6. Measurement of oxidative stress parameters:

Lipid peroxidation

In order to measure LPO, which is an important biomarker for oxidative stress, malondialdehyde (MDA) levels in the kidney and liver are detected and measured. This technique was established and developed by Jamall and Smith. This method is based on the reaction that takes place between MDA and thiobarbituric acid (TBA), which eventually ends with the development of a unique complex that is pink in color by preparing a mixture that includes a combination of 0.2 ml of kidney homogenate, 0.2 ml of a solution of sodium dodecyl sulphate (SDS) with a concentration of 8.1% to solubilize cellular components, 1.5 ml of a solution of 0.8% TBA to react with MDA, and 0.6 ml of water for dilution followed by incubation of the mixture in a water bath at a temperature of 95^{o} C for one hour. What confirms the success of the reaction is the unique pink colour. Following the incubation, 2 ml of 10% trichloroacetic acid (Sigma Aldrich, 27242) was added to 2 ml of the mixture and centrifuged at a speed of 1000 rpm for 10 minutes in order to separate the precipitate from the supernatant. The absorbance of the supernatant was measured at a wavelength of 532 nm using a spectrophotometer (Thermo Scientific Evolution 300, Waltham, MA). The MDA level in each sample was determined by utilizing the standard curve (Aydin et al, 2001). The MDA results were expressed as (nmol/g protein).

2.2.7. Measurement of antioxidative defense parameters:

Catalase Activity

The precise preparation of a phosphate buffer solution entails dissolving 2.57 grams of disodium phosphate and 1.29 grams of monosodium diphosphate in 500 ml of distilled water. The pH of the resultant mixture is meticulously adjusted to 7.0 using a solution of citric acid. Subsequently, 50 ml of the phosphate buffer is combined with 200 µL of 30% hydrogen peroxide (H_2O_2). Catalase (CAT) enzyme activity is assessed by meticulously preparing eight standard CAT solutions through the dilution of the enzyme in phosphate buffer. Spectrophotometric measurements at 240 nm are employed to evaluate the impact of varying H_2O_2 concentrations on CAT activity, which is then quantified in kU/g of protein (Aydin et al, 2001).

Glutathione peroxidase (GSH-Px) levels

The homogenates obtained from the kidneys and liver were diluted by a factor of seventeen before the experiment was carried out. To create a combination of 1 ml in a cuvette, a total of 10 µL of the samples were combined with 990 µL of the reaction mixture. This allowed for the creation of a mixture. In the following step, the mixture was set aside at room temperature for a period of five minutes. Following the introduction of 10 µL of tert-butyl hydroperoxide, the experimental process proceeded. Subsequently, the reduction in NADPH absorbance was measured at a wavelength of 340 nanometers. This measurement was carried out over the course of three minutes, and seven measurements were taken in total during that time. This is done in order to determine the rate of change in absorbance per unit of time. GSH-Px activity was measured and reported in units per gram of protein (U/g protein). This quantitative analysis was carried out (Aydin et al, 2001).

2.2.8. Histopathology analysis:

After the experimental animals were sacrificed, the kidney tissue was fixed in a 10% formaldehyde solution. The tissues were then subjected to routine histological evaluations, dehydrated in ascending alcohol series, made transparent in xylene, and then embedded in paraffin. 5 µm thick sections were taken from the paraffin-blocked tissues and stained with hematoxylin and eosin (H&E) for histopathological evaluation. H&E-stained sections were scored semi-quantitatively for tubulointerstitial damage. Scoring criteria were evaluated in 6 parameters as "tubular dilatation", "tubular desquamation", "tubular vacuolization", "intratubular vascular congestion", "intratubular inflammation" and "hyaline cast". Using these parameters, a score from 0 to 3 was given: 0 (no damage), 1 (mild), 2 (moderate), 3 (severe).

2.2.9. Statistical Analysis:

The statistical analysis was performed by (IBM SPSS, version 27). One-way analysis of variance was used followed by Dunnett’s T3 Post Hoc Test in order to perform comparisons among all different groups. $P < 0.05$ is considered statically significant.

3. Results

3.1. Blood Analysis:

Before the animals had been sacrificed, blood samples were collected and analyzed in order to observe if there is any abnormal effect on the blood among the experimental groups. The blood test did not show any significant abnormality among the groups.

Table 3 Results of blood analysis

Group		WBC (10^3/µL)	LYM (%)	MID (%)	GRAN (%)	LYM# (10^3/µL)	MID# (10^3/µL)	GRAN# (10^3/µL)
Control	Mean	13.5429	61.8857	11.3429	26.7429	8.5714	1.5286	3.4429
	SD	6.64351	4.58746	1.34022	4.28558	4.78216	0.69213	1.23404
Cisplatin	Mean	12.6857	40.0143	11.7	48.2571	4.9286	1.5143	6.2429
	SD	6.21515	14.91805	3.0795	17.07463	3.15263	0.91183	3.67372
Cis-NAC	Mean	17.7571	42.8	11.2429	45.9571	7.5429	2.0571	8.1571
	SD	4.99962	13.20063	3.37187	15.56822	3.0967	0.94315	3.27305
Cis-WP	Mean	12.9429	50.2429	11.4571	38.3	6.4429	1.4714	5.0286
	SD	4.17367	13.82556	2.97313	14.26768	2.5566	0.47509	2.87617
Cis-NAC-WP	Mean	9.7286	45.3143	13.3571	41.2714	4.5571	1.3429	3.8286
	SD	3.6188	7.1913	2.50989	8.74714	1.85998	0.63994	1.25527

RBC ($10^6/\mu L$)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	ROW-SD (fL)	ROW-CV (%)	PLT ($10^3/\mu L$)	MPV (fL)	PWD (fL)	PCT (%)
8.06	18.7143	50.6714	62.8429	23.1143	36.7857	36.9	17.3429	553.7143	8.3429	10.4143	0.46
0.78064	2.59193	5.90387	1.83744	1.19921	1.4206	1.66733	0.62412	207.7865	0.33594	0.564	0.18956
6.5786	15.3143	40.6429	61.7571	22.9	37.3143	35.0429	16.6714	473.5714	8.2857	11.1571	0.3857
1.99383	5.32836	12.57893	0.93783	1.44338	2.43134	2.74885	1.16578	213.5032	0.6283	2.93817	0.17662
7.8386	18.6143	47.9429	61.1286	23.6429	38.7143	36.6286	17.4857	522.5714	8.2857	10.9571	0.4329
0.68635	2.45793	5.22489	1.84184	1.52081	1.63445	2.7693	1.63649	155.9133	0.5757	1.96117	0.1458
7.6843	18.7286	47.8143	62.1571	24.5143	39.5429	36.9286	17.6143	606.1429	8.3143	10.8429	0.5029
1.73954	3.95125	11.10427	2.48654	1.39215	2.92396	0.71813	0.69625	198.4997	0.46701	1.1341	0.1875
7.3843	17.7286	46.0714	62.3429	23.6286	38	35.8429	16.9143	511.5714	8.1857	11.9286	0.4186
2.22566	**5.74042**	**14.01448**	**1.01136**	**1.40679**	**1.90088**	**2.99492**	**1.25887**	**242.2656**	**0.45617**	**1.40679**	**0.20932**

PLCR (%)	4.3714	1.41623	3.6286	0.96387	5.3143	2.0619	4.3571	0.79132	3.8429	**0.49952**

3.2. Organ body weight ratio:

There was no significant body weight change between the initial and last body weight among all groups. The body weight of all groups has been reduced compared to the control group. However, this reduction was not statistically significant due to the high heterogeneity of animal body weight initially. On the other hand, there was no significant change in kidney weight and kidney body weight ratio.

All the groups show statistically significantly lower liver weight compared to the control group. On the other hand, the liver-body weight ratio was significantly less in Cis-NAC-WP compared to the control group. The last body weight, liver weight and liver/body weight ratio (%). All data is summarized in table 4.

Table 4 Changes in Body Weight and Organ Ratios

Group	Initial body weight (g)	Last body weight (g)	Kidney weight (g)	Liver weight (g)	Kidney/body weight ratio (%)	Liver/body weight (%)
Control	**258.71 ± 30.37**	**263.71 ± 28.50**	**2.69 ± 0.27**	**10.85 ± 0.86**	**1.03 ± 0.18**	**4.13 ± 0.32**
Cisplatin	**257.28 ± 23.99**	**235.28 ± 39.81**	**2.49 ± 0.25**	**7.64 ± 1.16[a]**	**1.08 ± 0.19**	**3.30 ± 0.59**
Cis-NAC	**241.71 ± 36.84**	**242.85 ± 38.83**	**2.52 ± 0.45**	**7.55 ± 1.45[a]**	**1.06 ± 0.28**	**3.61 ± 0.26**
Cis-WP	**232.28 ± 21.19**	**224.71 ± 36.55**	**2.37 ± 0.30**	**8.14 ± 1.57[a]**	**1.07 ± 0.20**	**3.15 ± 0.32**
Cis-NAC-WP	**251.57 ± 7.89**	**220.28 ± 17.32**	**2.54 ± 0.17**	**6.92 ± 0.65[a]**	**1.15 ± 0.05**	**3.46 ± 0.53[a]**

Values are mean ± standard deviation (n =7). Data were analyzed using one-way ANOVA followed by Dunnett’s T3 test.

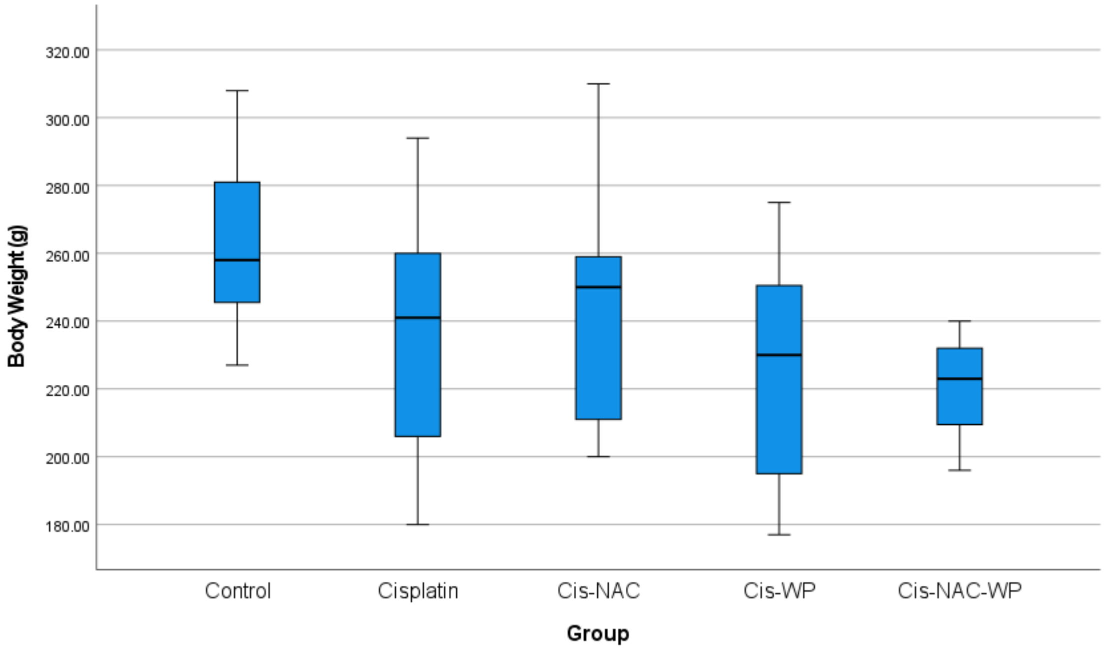

Figure 4 Body weight changes among all group

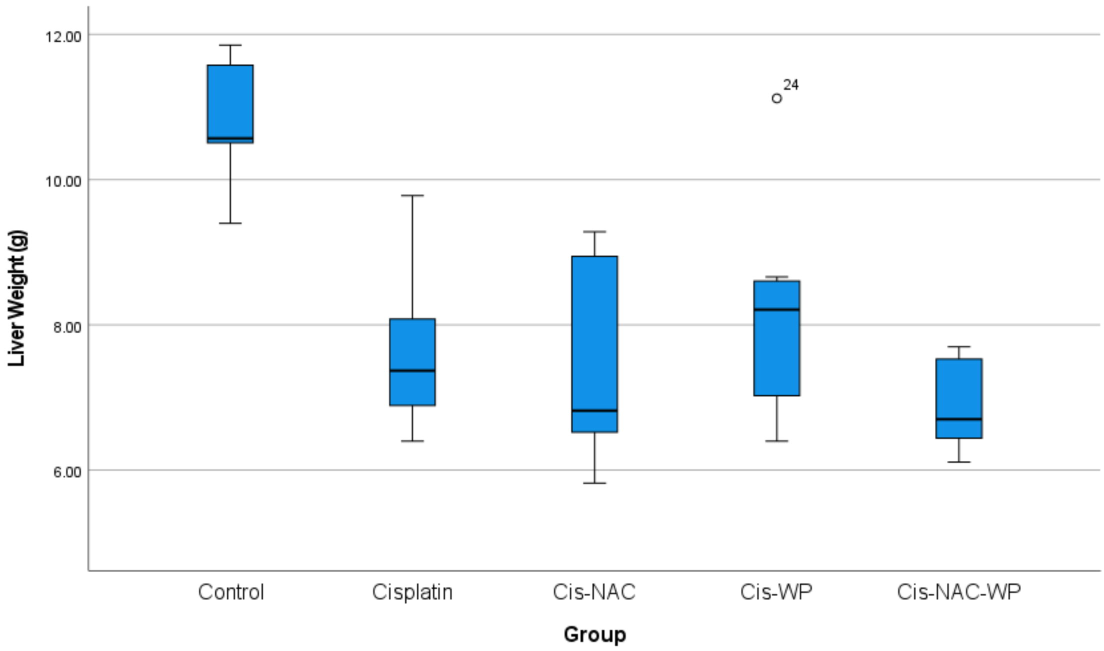

Figure 5 Liver weight results among all groups

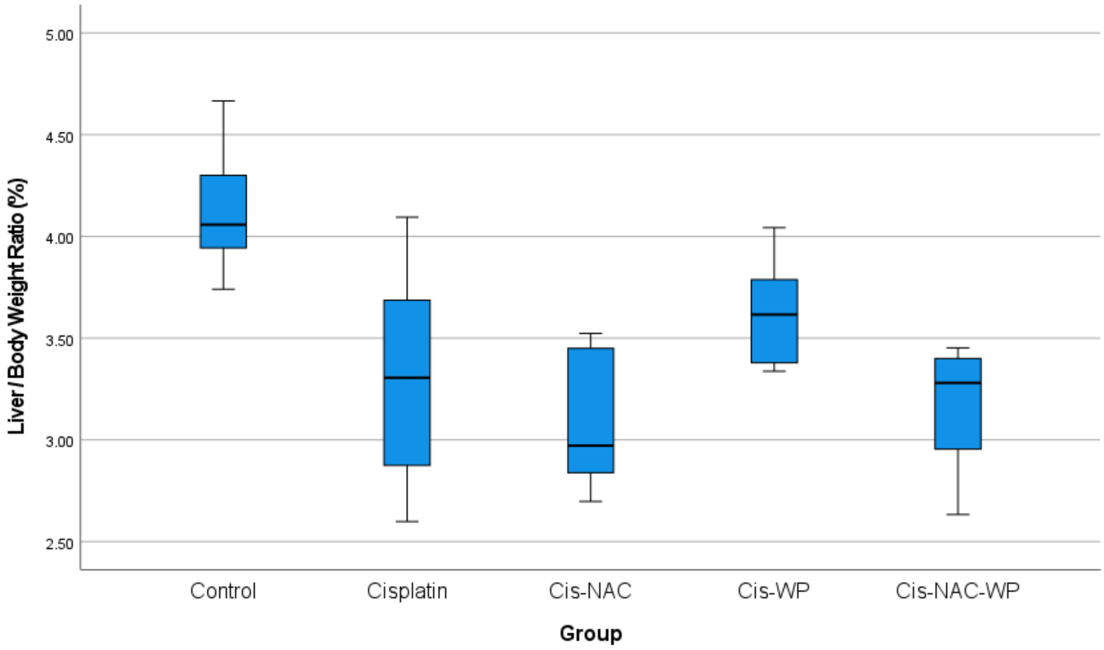

Figure 6 Liver/body weight ratio (%)

3.3. Biochemical Analysis

For the kidney, the urea level in serum in CIS-NAC-WP group was significantly decreased compared to the cisplatin group. The cisplatin group shows significantly a higher urea level compared to the control group. However, the other groups did not show any statistically significant change. Similar results were found in serum creatinine in addition to a significant decrease of serum creatinine levels between Cis-WP and cisplatin group.

For the liver, the ALT and AST did not show any significant change among all the groups. The following table illustrates the results of biochemistry analysis.

Table 5 The effect of Whey protein and N- acetylcysteine on serum urea, creatine, ALT and AST against cisplatin-induced toxicity

Group	Urea (mg/dL)	Creatinine (mg/dL)	ALT (U/L)	AST (U/L)
Control	41.14 ± 4.05	0.38 ± 0.05	49.14 ± 9.38	169.28 ± 84.23
Cisplatin	283.28 ± 148.87[a]	2.43 ± 1.44[a]	51.57 ± 20.64	143.71 ± 23.32
Cis-NAC	96.28 ± 47.53	0.91 ± 0.43	51.85 ± 9.94	125.28 ± 27.09
Cis-WP	89.85 ± 58.00	0.77 ± 0.51[b]	38.71 ± 7.96	116.71 ± 13.82
Cis-NAC-WP	44.14 ± 10.99[b]	0.43 ± 0.08[b]	50.14 ± 12.07	148.28 ± 48.14

Values are mean ± standard deviation (n =7). Data were analyzed using one-way ANOVA followed by Dunnett’s T3 test.

a Statistically significant compared with control group ($P < 0.05$).

b Statistically significant compared with CP group ($P < 0.05$).

c Statistically significant compared with CP-NAC group (P < 0.05).
d Statistically significant compared with CP-WP group (P < 0.05).

3.4. Oxidative Stress in Kidney

MDA Levels in Kidney

The kidney MDA levels in the cisplatin group have been significantly increased compared to all other groups. On the other hand, the groups that have nephroprotective agents such as Cis-NAC, Cis-WP and Cis-NAC-WP show decreased MDA levels in rat kidney tissue compared to Cisplatin group (P-value < 0.05). Figure 7 illustrates the difference in MDA levels among all groups. All the results of oxidative and antioxidative stress parameters are summarized in Table 6.

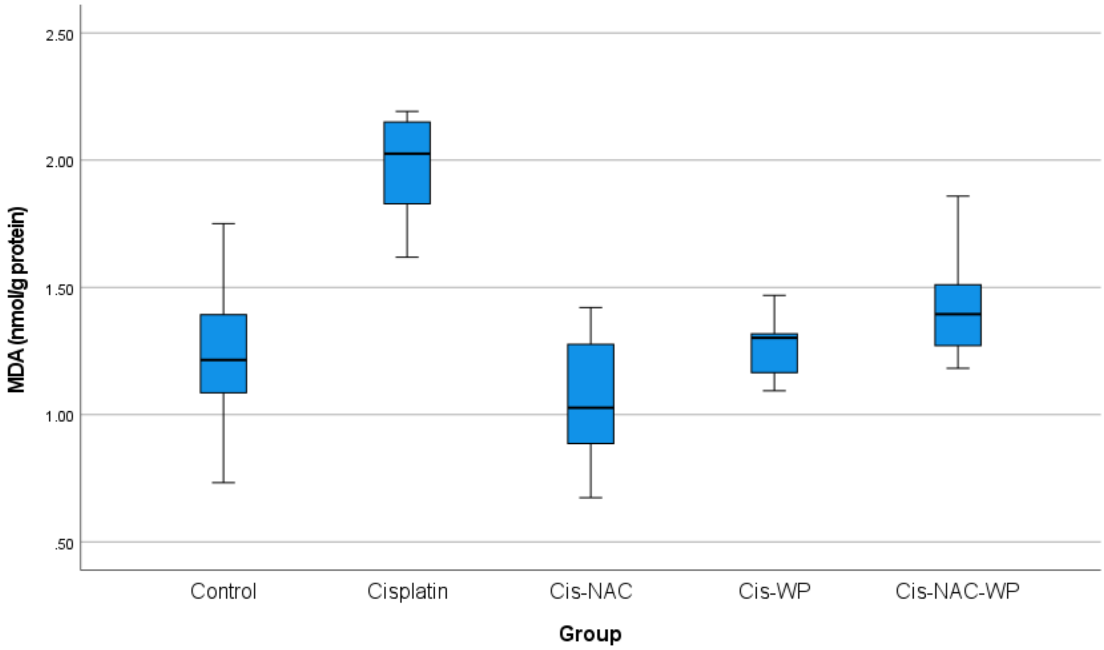

Figure 7 The effect of nephroprotective agents and cisplatin on MDA levels in kidney

3.5. Antioxidant Parameters in Kidney

GSH-Px Activity in Kidney

Cis-NAC-WP group shows a significantly higher GSH-Px activity in comparison to all groups excluding Cis-NAC p < 0.05, Figure 8). On the other hand, there are no significant differences among the other groups.

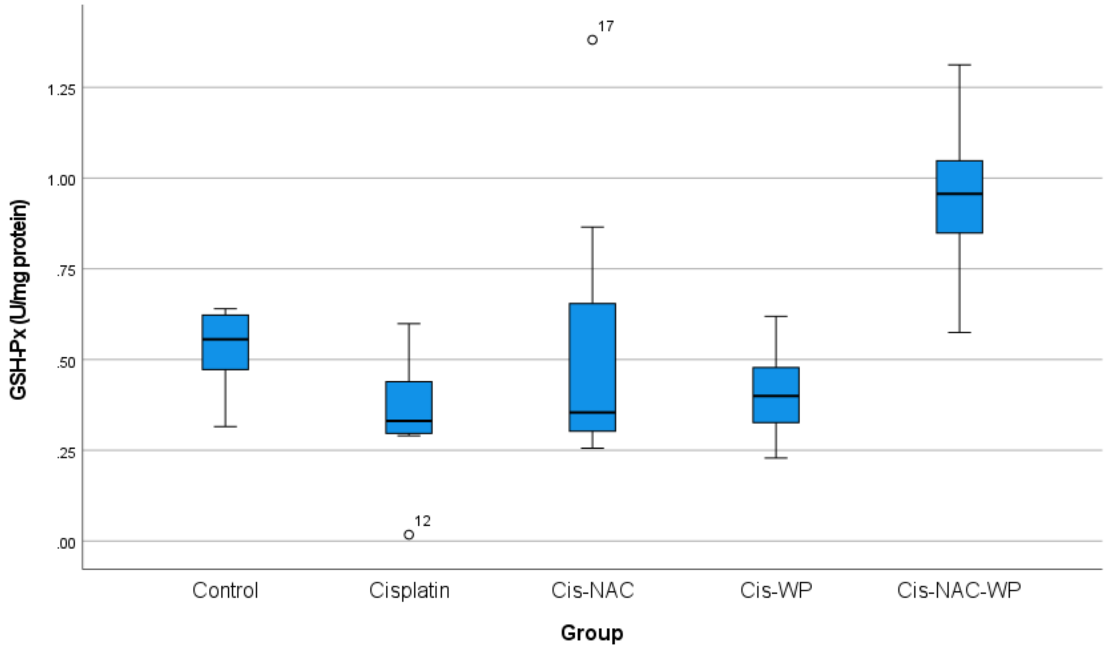

Figure 8 The effect of nephroprotective agents and cisplatin on GSH-Px levels in kidney

CAT Activity in Kidney

There is no significant difference among all groups in terms of CAT activity change in the kidneys ($p > 0.05$, Figure 9). However, the control and nephroprotective groups seem to have a higher CAT activity compared to the cisplatin group.

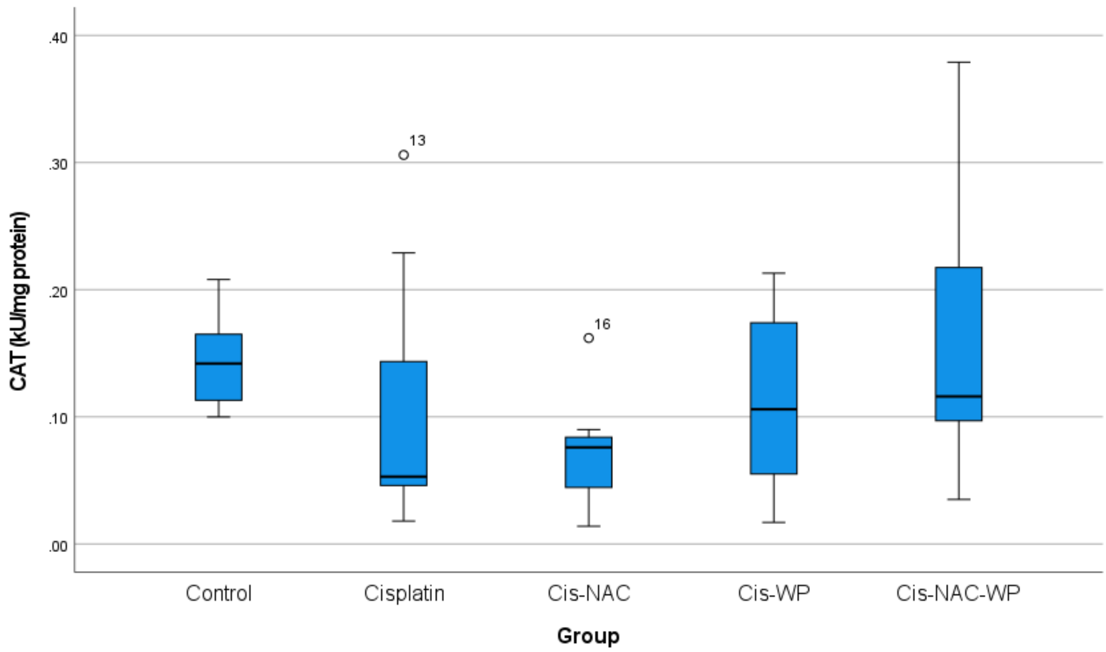

Figure 9 The effect of nephroprotective agents and cisplatin on CAT levels in kidney

Table 5 Results of oxidative stress and antioxidant parameters in kidney

Group	MDA (nmol/g protein)	GSH-Px (U/mg protein)	CAT (kU/mg protein)
Control	1.24 ± 0.32	0.53 ± 0.12	0.14 ± 0.04
Cisplatin	1.97 ± 0.22*	0.35 ± 0.18	0.11 ± 0.11
Cis-NAC	1.06 ± 0.27^{b}	0.56 ± 0.42	0.07 ± 0.05
Cis-WP	1.26 ± 0.13^{b}	0.41 ± 0.13	0.11 ± 0.08
Cis-NAC-WP	1.43 ± 0.23^{b}	0.95 ± 0.23^{abd}	0.17 ± 0.13

Values are mean ± standard deviation (n =7). Data were analyzed using one-way ANOVA followed by Dunnett's T3 test.

a Statistically significant compared with control group (P < 0.05).

b Statistically significant compared with CP group (P < 0.05).

c Statistically significant compared with CP-NAC group (P < 0.05).

d Statistically significant compared with CP-WP group (P < 0.05).

***** Statistically significant compared with all groups (P < 0.05).

3.6. Oxidative Stress and Antioxidative Parameters in Liver

MDA Levels in Liver

Malondialdehyde (MDA): Our study quantified MDA levels, a biomarker for oxidative stress, in several groups. The cisplatin group had elevated levels of MDA in comparison to the control group. however, the combination group Cis-NAC-WP show a significantly lower activity of MDA levels compared to the cisplatin group, indicating a lower lipid peroxidation activity in this group.

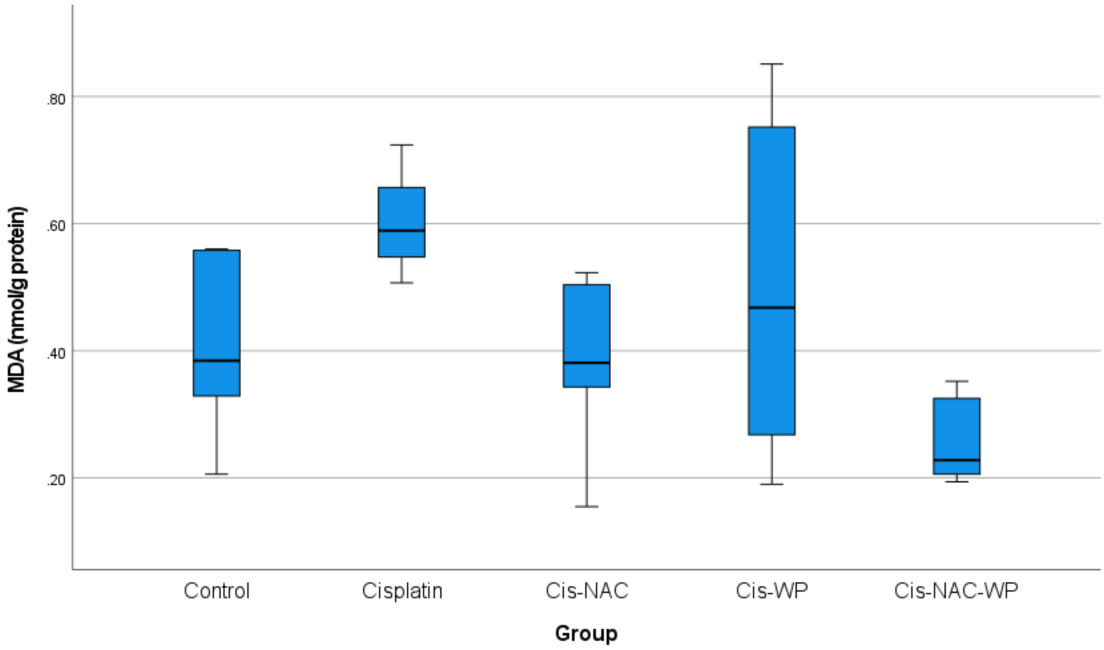

Figure 10 The effect of nephroprotective agents and cisplatin on MDA levels in liver

GSH-Px Levels in Liver

GSH-Px was examined as a significant antioxidant enzyme. The cisplatin group exhibited no substantial disparity in GSH-Px levels as compared to the control group. Nevertheless, in comparison to the CP-WP and CP-NAC-WP treatments, there was an observable, if not statistically significant, rise in GSH-Px levels. These findings indicate that CP-WP and CP-NAC-WP may strengthen the body's antioxidant defence, so offering a degree of protection against the oxidative damage induced by cisplatin.

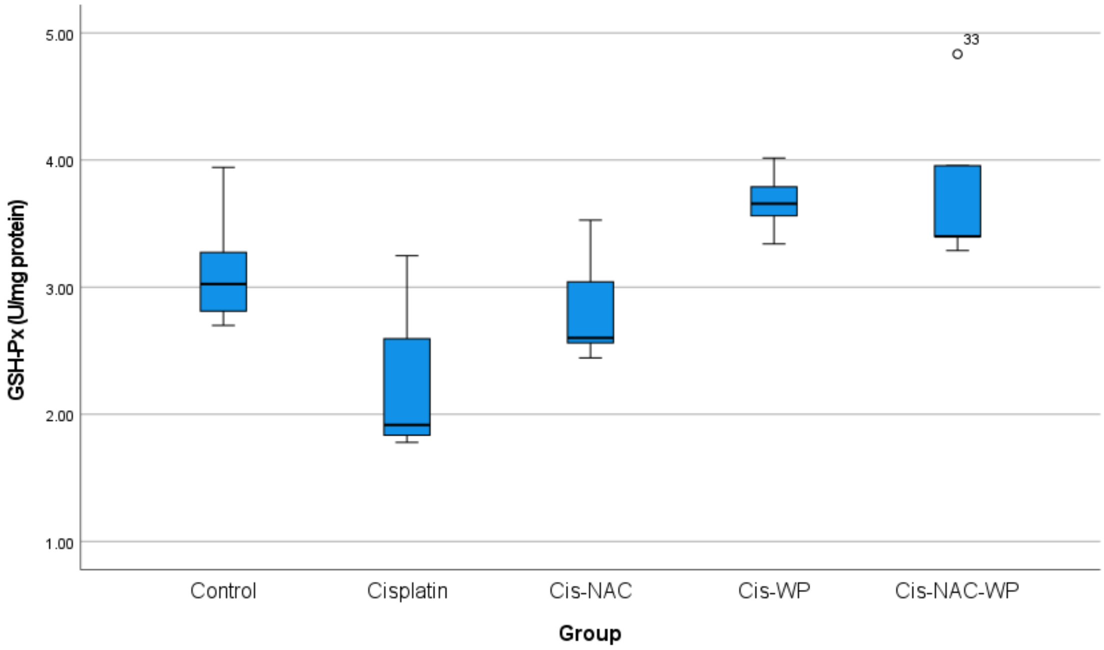

Figure 11 The effect of nephroprotective agents and cisplatin on GSH-Px levels in liver

CAT levels in Liver

There were no significant changes in CAT levels across all groups, including the control, cisplatin, and the other treatments with nephroprotective agents (NAC, WP or both combined).

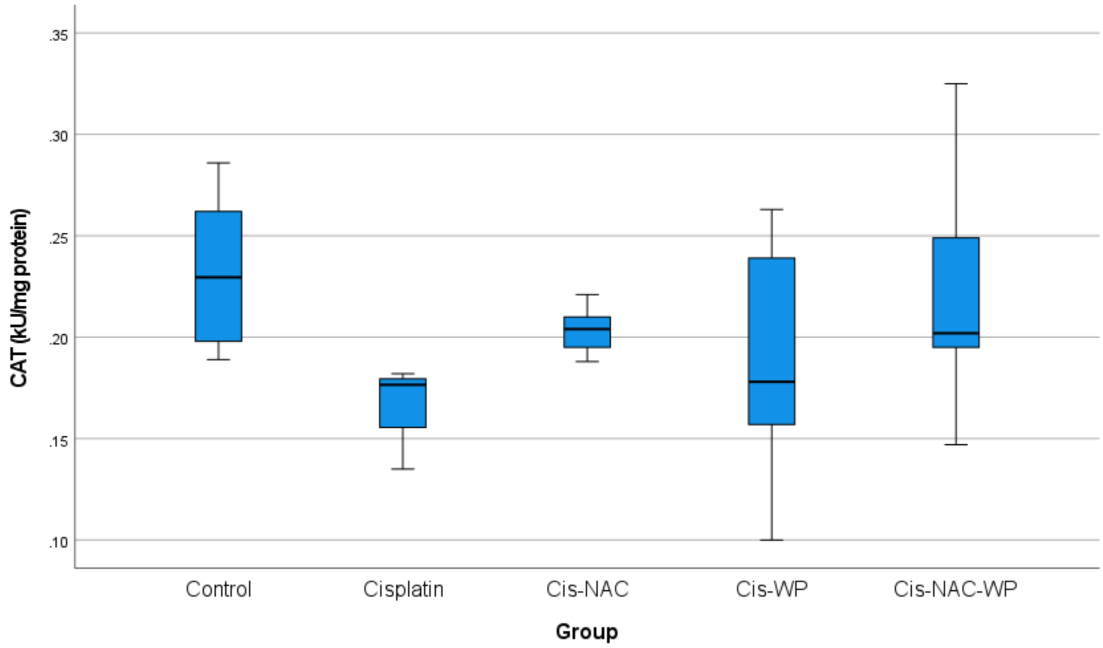

Figure 12 The effect of nephroprotective agents and cisplatin on CAT levels in liver

Table 6 Results of oxidative stress and antioxidant parameters in liver

Group	MDA (nmol/g protein)	GSH-Px (U/mg protein)	CAT (kU/mg protein)
Control	0.40 ± 0.13	3.13 ± 0.46	0.24 ± 0.04
Cisplatin	0.59 ± 0.14	2.22 ± 0.69	0.21 ± 0.08
Cis-NAC	0.47 ± 0.19	2.84 ± 0.45	0.22 ± 0.03
Cis-WP	0.46 ± 0.26	3.67 ± 0.23	0.19 ± 0.06
Cis-NAC-WP	0.27 ± 0.07[b]	3.78 ± 0.65	0.23 ± 0.06

Values are mean ± standard deviation (n =7). Data were analyzed using one-way ANOVA followed by Dunnett's T3 test.

a Statistically significant compared with control group (P < 0.05).

b Statistically significant compared with CP group (P < 0.05).

c Statistically significant compared with CP-NAC group (P < 0.05).

d Statistically significant compared with CP-WP group (P < 0.05).

3.7. Histopathology:

When the cortex and medulla of the kidney sections of the control group (control) were examined under a light microscope, the glomerulus, Bowman's capsule, proximal and distal tubules, collecting tubes and vessels were observed to have a normal structure. Although regular tubulointerstitial areas were observed, no pathological findings were found (Figure 13).

Tubular dilatation (expansion) is noteworthy in the group given cisplatin. These tubules were observed as oval and spiral-shaped structures with highly dilated lumens continuing from the cortex to the medulla. Desquamated cells (shedding of epithelial cells into the lumen) were observed occasionally within the dilated tubules. Additionally, in addition to tubular dilatation, hyaline cast (eosinophilic stained structures) accumulation was observed in the lumen of these tubules. It has been observed that the epithelial cells lining the proximal tubules exhibit advanced degenerative features. In the cytoplasm of the epithelial cells lining these tubules; It was determined that the nuclei could not be distinguished, there was desquamation and cytoplasmic vacuolization (foamy appearance in the cytoplasm). Numerous leukocytic infiltrates were observed in the cortex and corticomedullary area. It has been determined that in most renal corpuscle structures, Bowman's space becomes irregular and widened due to the shrinkage of the glomerular structure. It has been observed that the glomerular structure in the renal corpuscle, where there is leukocyte infiltration, is degenerated. Erythrocyte extravasation was detected in tubuloinsterstitial areas in the cortex and medulla (Figure 14).

In the groups given Cis-WP and Cis-NAC; When kidney sections were examined, it was observed that the cortex structure had a more normal histological appearance compared to the group given only cisplatin. Although there was a significant decrease in tubular dilatation, it was determined that tubular vacuolization, desquamation, vascular congestion, inflammation and hyaline cast structures were at a mild level (Figure 15, Figure 16).

In the group given Cisplatin, Cis-NA-WP; In contrast to cisplatin-induced degeneration, proximal tubules with normal morphology lined by many acidophilic cells were observed in the cortex of kidney sections in this group. The nuclei of the cells surrounding most tubules were seen as round. Most of the renal corpuscle structure was observed to be close to normal. It was observed that the glomerular structure became smaller and the Bowman space gained a near-normal appearance. It was determined that there was a significant decrease in hyaline cast structures and inflammation in the cortex and medulla of the kidney sections of this group. Morphological findings similar to the control group were observed (Figure 17).

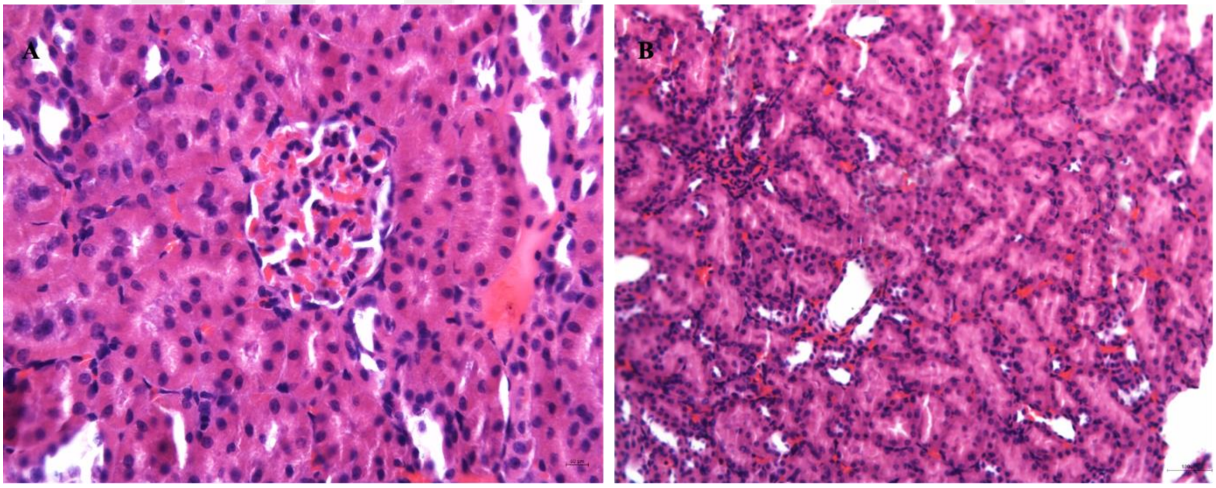

Figure 13 Kidney tissue of the Control group. A. Cortex region B. Medulla region

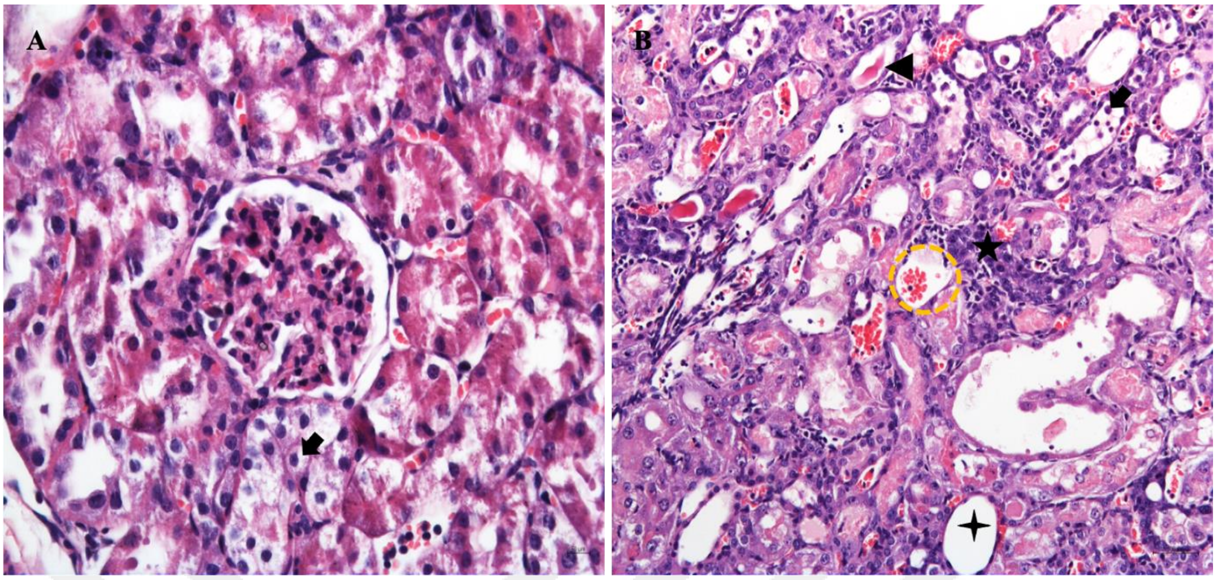

Figure 14 Kidney tissue belonging of Cisplatin Group. A. Cortex region. B. Medulla region.

Kidney tissue belonging to (Cisplatin Group). A. Cortex region tubular vacuolization (black arrow) B. Medulla region tubular desquamation (black arrow), hyaline cast (arrowhead), tubular dilatation (four-pointed star), leukocyte infiltration (star), vascular congestion (yellow circle)

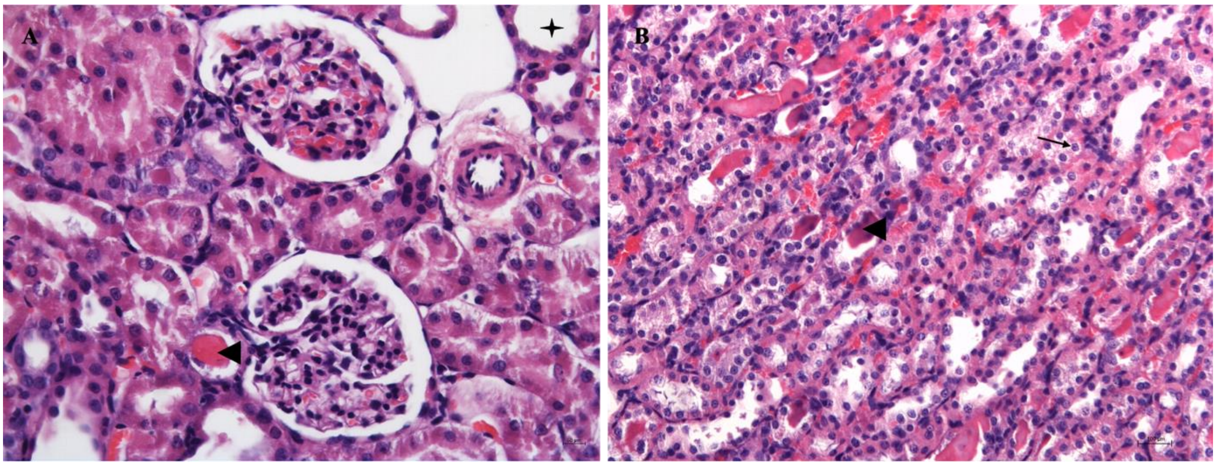

Figure 15 Kidney tissue belonging of Cis-NAC Group. A. Cortex region. B. Medulla region.

Kidney tissue belonging to (Cis-NAC) group. A. Cortex region, mild hyaline cast (arrowhead), tubular dilatation (four-pointed star) B. Medulla region, mild tubular vacuolization (black arrow), hyaline cast (arrowhead).

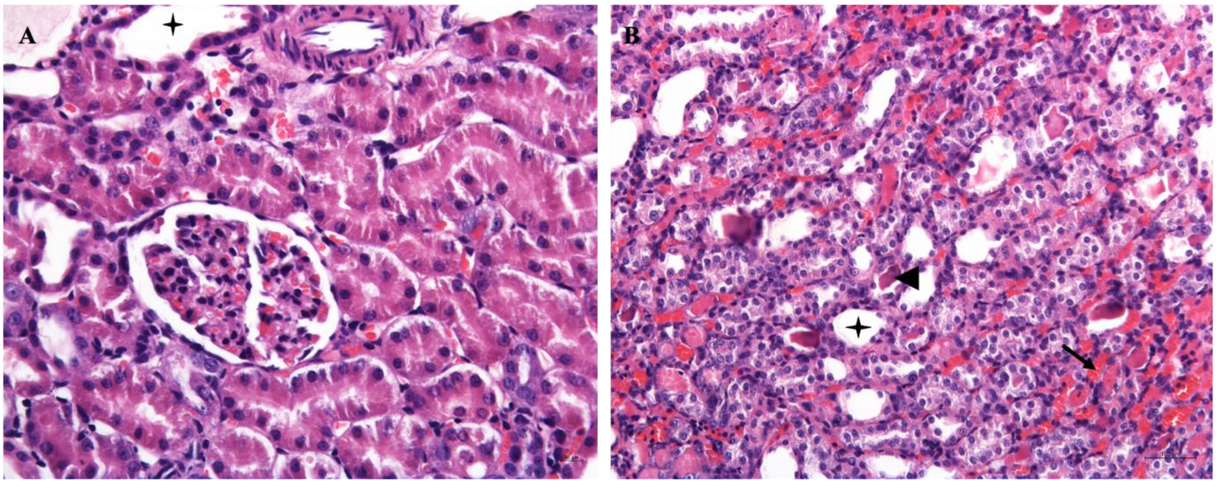

Figure 16 Kidney tissue belonging of Cis-WP Group. A. Cortex region. B. Medulla region.

Kidney tissue belonging to Cis-WP group. A. Cortex region mild tubular dilatation (four-pointed star) B. Medulla region mild tubular dilatation (four-pointed star), hyaline cast (arrowhead), vascular congestion (black arrow)

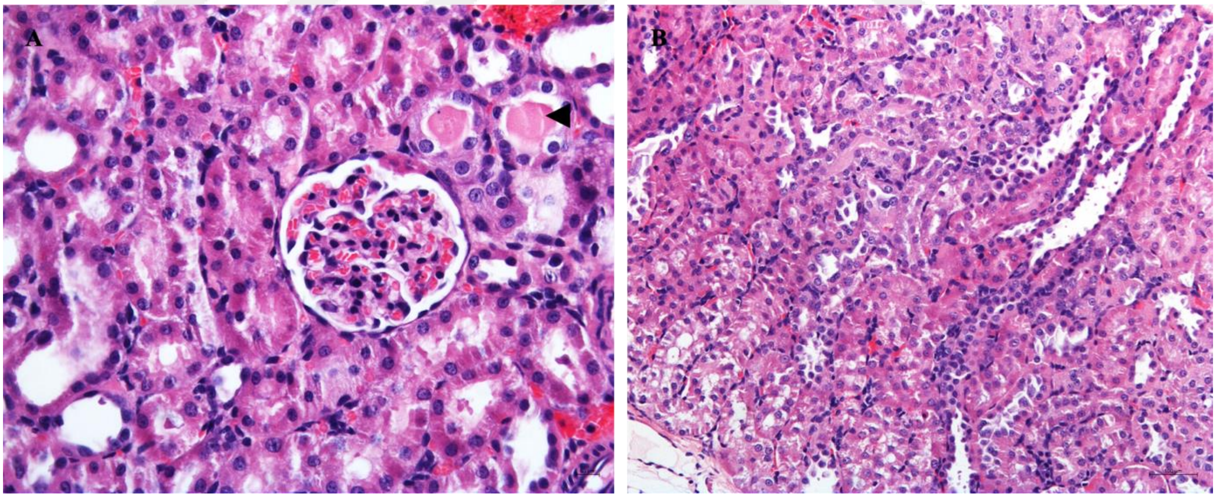

Figure 17 Kidney tissue belonging of Cis-NAC-WP Group. A. Cortex region. B. Medulla region.

Kidney tissue belonging to Cis-NAC-WP group. A. Cortex region Hyaline caste (arrowhead) in the distal tubule B. Medulla region

Histopathological semi-quantitative analysis

Cisplatin treatment caused a significant increase in tubular dilatation. When we compared it to the CP-NAC group, we found a notable difference, with cisplatin causing more dilatation by a margin of 2.0 units on our scale ($p < 0.05$). The difference was slightly higher when comparing cisplatin to the CP-WP group, with a margin of 2.14 units ($p < 0.05$). The most significant difference has been observed between cisplatin and CP-WP-NAC, where the margin was 2.71 units ($p < 0.05$).

Cisplatin also caused more tubular desquamation compared to all other groups. The elevation was significant when compared to the CP-NAC group, with a difference of 1.71 units ($p = 0.001$). This effect was even more pronounced when comparing cisplatin to CP-WP and CP-WP-NAC, with differences of 1.86 units and 2.43 units, respectively (both $p < 0.05$).

We observed that cisplatin significantly increased tubular vacuolization. Compared to CP-NAC, the difference was 2.0 units ($p = 0.001$). The difference was similar when compared to CP-WP, at 2.14 units ($p < 0.05$). Against CP-WP-NAC, the increase was again notable at 2.71 units ($p < 0.05$).

Cisplatin led to significant intratubular vascular congestion. It caused more congestion compared to CP-NAC by 2.0 units ($p < 0.05$). The increasement was similar compared to CP-WP with a difference of 2.14 units ($p < 0.05$). When comparing cisplatin to CP-WP-NAC, the congestion was significantly higher by 2.57 units ($p < 0.05$).

Cisplatin treatment also resulted in increased intratubular inflammation. The difference compared to CP-NAC was 2.0 units ($p < 0.05$). Comparing cisplatin to CP-WP, the increase was 2.14 units ($p < 0.05$). The most significant increase was noted when compared to CP-WP-NAC with a difference of 2.43 units ($p < 0.05$).

Lastly, cisplatin treatment significantly increased the accumulation of hyaline substances in the kidney tubules. Compared to CP-NAC, the increase was 1.86 units ($p < 0.05$). The difference was 2.0 units when comparing cisplatin to CP-WP ($p < 0.05$). The accumulation was highest when comparing cisplatin to CP-WP-NAC, with a difference of 2.29 units ($p < 0.05$). The data summary of semi-quantitative analysis is presented in Table .

Table 7 Scored semi-quantitatively analysis of histopathological results of the kidney among all groups

Group	Tubular Dilatation	Tubular Desquamation	Tubular Vacuolization	Intratubular Vascular Congestion	Intratubular Inflammation	Hyaline Substance Accumulation
	Mean	Mean	Mean	Mean	Mean	Mean
Control	0	0	0	0	0	0
Cisplatin	3	2.7	3	3	3	2.7
Cis-NAC	1	1	1	1	1	0.87
Cis-WP	0.85	0.85	0.86	0.86	0.86	0.71
Cis-NAC-WP	0.29	0.29	0.29	0.43	0.57	0.43

4. Discussion and Conclusion

The primary objective of this study is to examine the effects of cisplatin and its combination with NAC, WP, or both substances on a range of physiological, histological, biochemical and molecular indicators in rats. The hematological evaluation shows no significant abnormalities in any of the experimental groups. Thus, according to the results may be deduced from this that the therapies which were administrated did not result in any haematological toxicity throughout the observation period, which suggests that there is no observable regarding the systemic blood parameters. These findings which are related to the hematological parameters are not reliable due to the short duration of this experiment.

The results of the current research showed that there were no significant changes in body weight from the initial to the final measures across the groups. This lack of change can be attributed to the large heterogenicity in body weight among all rats in all groups. While the experimental groups showed a decrease in body weight relative to the control group, the wide range of beginning body weights made these changes statistically inconsequential. The kidney weight and kidney/body weight ratio exhibited no statistically significant variations across the groups, suggesting that the therapies did not induce any renal hypertrophy or atrophy.

Significant findings were observed in the examination of liver weight and liver/body weight ratio. Each of the groups that received treatment had considerably lower liver weights in comparison to the control group. Furthermore, the Cis-NAC-WP group demonstrated a notably lower liver-body weight ratio. This indicates a possible harmful effect on the liver or a decrease in liver size which may be caused by the therapeutic effects of the combination therapy. The decrease in liver body weight ratio might be linked to the defensive properties of NAC and WP against cisplatin-induced hepatotoxicity. This reduction of liver/body weight ratio is explained before by (Garcia et al., 2013). According to their findings, liver weight reduction in rodents occurs due to cachexia and the loss of lipids that are induced by cisplatin, but it is not possible to confirm the change of the organ or body weight in this experiment due to the high heterogenicity of the initial body weight of the used rats. However, the dose of cisplatin which is used in the current experiment is nephrotoxic rather than hepatotoxic.

Histopathological examination of kidney tissues provided crucial insights into the structural impacts of the treatments. The control group had typical renal structure without any abnormal observations. On the other hand, the cisplatin group exhibited notable expansion of the tubules, shedding of cells, formation of vacuoles, buildup of hyaline casts, infiltration of white blood cells, and reduction in the size of the glomeruli, all of which are indicative of nephrotoxicity caused by cisplatin. The groups who received Cis-NAC, Cis-WP, and Cis-NAC-WP exhibited significant enhancements in renal histopathological profile when they were compared with the cisplatin group. Particularly, the Cis-NAC-WP group had renal histology that was close to normal. This suggests a synergistic protective effect of NAC and WP against cisplatin-induced kidney damage. Several studies have shown similar histological results for the protective effect of NAC against cisplatin-induced nephrotoxicity. Findings show that the histological architecture of the NAC groups is better than the solely cisplatin-treated group [30, 37, 192, 216 – 218].

Oxidative stress in the kidneys was assessed by measuring MDA levels, which were significantly higher in the cisplatin group compared to the other groups. A significant reduction in MDA levels was seen as a consequence of treatments that included either NAC, WP, or a combination of both agents. There is a decrease in the amount of oxidative stress which is present inside the kidneys. This combination has the potential to be beneficial in lowering oxidative damage, as evidenced by the fact that the Cis-NAC-WP group demonstrated the most substantial drop. As a result of the combination therapy, the Cis-NAC-WP group exhibited considerably raised levels of antioxidant indices, including GSH-Px activity. This indicates that the antioxidant defenses in the kidney tissues have been greatly enhanced. CAT activity did not show any significant differences between the groups. However, the CAT levels were slightly decreased in cisplatin group, and increased in the groups that received nephroprotective agents such as NAC and WP. The increase were almost following same manner in both kidney and liver where the combination of WP and NAC in Cis-NAC-WP group seems to have a higher activity compared with other groups which received WP or NAC solely.

Oxidative stress was shown by the higher levels of MDA in the liver of the cisplatin group compared to the control. The CP-NAC-WP group exhibited a significant decrease in MDA levels in comparison to the group treated with cisplatin alone, highlighting its ability to protect against oxidative damage in the liver. The levels of GSH-Px in the liver were elevated in the CP-WP and CP-NAC-WP groups, indicating an improved antioxidant response. However, there were no significant alterations in CAT levels across all the groups and this is a normal result because CAT and GSH-Px work on reducing the same free radicals. Therefore, it is not necessary for both enzymes to be enhanced correlatively

Several research investigated the effects of NAC on inflammation and oxidative stress in cisplatin-induced nephrotoxicity in rats, finding that NAC significantly reduced oxidative stress and inflammation markers. Their findings showed that NAC significantly reduced nephrotoxicity by the reduction of oxidative stress and inflammatory biomarker and increasing the activity of antioxidant enzymes such CAT, SOD and GSH-Px, [192, 195, 217 – 218]. This is consistent with our results, where NAC and WP reduced oxidative stress markers and improved antioxidant enzyme levels in both kidney and liver tissues enhancing intracellular cysteine levels, boosting glutathione GSH production, and providing substantial protection against oxidative damage, correlating with our findings of enhanced GSH-Px activity in the NAC and WP treated groups. Further support comes from Dickey et al. (2007), who investigated the effects of different routes of NAC administration on chemoprotection against cisplatin-induced toxicity in rat models [31]. Their emphasizing the importance of administration routes for optimizing NAC's protective effects [31]. Another study highlighted the synergistic protective effects of lycopene and NAC against cisplatin-induced nephrotoxicity, significantly improving renal function and reducing oxidative stress markers [218]. This supports the potential benefits of combining antioxidant therapies, as demonstrated in our study with NAC and WP

In the current study, the renal protection was not limited to oxidative stress parameters and histopathological alternation only. Another synergistic effect was obvious on the biochemical level whereas the administration of WP and NAC along with CP shows a decrease in the serum levels of creatinine and urea. the combination of WP and NAC in Cis-NAC-WP group showed a reduction in the serum levels of urea and creatinine which suggest a superior synergistic effect. There are several studies show that NAC can decrease the serum levels of urea and creatinine. However, one study found that lactoferrin - which is one of whey protein fractions - has the ability to decrease the same kidney function biochemical parameters. Decreasing the nephrotoxicity is not the only promising aspect of WP, it is also beneficial against cancer cachexia which enhances the quality of life of cancer patients who undergo cisplatin chemotherapy as has been shown in a previous case report [219].

There are several limitations in this study. The duration of the experiment was relatively short. It is recommended to be longer and go on with chemotherapy cell cycles and have repeated doses. On the other hand, more parameters must be studied in order to understand the mechanism behind the protective effect of whey protein. For example, the SOD was absent in the experiment. Inflammatory biomarkers must also be studied in order to evaluate the anti-inflammatory effect of whey protein. Although, the current study did not focus on the inflammatory biomarker due to limiting the scope on oxidative stress changes, the histological investigation revealed a notable intratubular inflammation was in Cisplatin group that was getting reduced in the other groups which received nephroprotective agents such as NAC and WP. However, the semi-qualitative results shows that the combination of NAC and WP in Cis-NAC-WP group has the lowest observable intratubular inflammation.

Overall, the study demonstrates that the combined treatment of NAC and WP significantly reduces cisplatin-induced nephrotoxicity and oxidative stress in both kidney and liver tissues. Histopathological analysis and oxidative stress markers consistently support the potential of NAC and WP in mitigating cisplatin-induced damage, underscoring the importance of further exploration of these agents as adjunct therapies in cisplatin-based treatments to enhance therapeutic outcomes and reduce side effects.

In conclusion, these findings revealed a positive protective effect of NAC and WP against cisplatin-induced nephrotoxicity and hepatotoxicity by decreasing the oxidative stress that occurs due to LPO, and enhancing the activity of enzymatic antioxidants such as GSH-Px in kidney tissue. Moreover, the combination of NAC and WP shows a synergistic protective effect on the histological architecture of renal tissue in addition to enhancing the biomarkers of kidney injury such as urea and creatinine. The limitation of this study is the duration of the experiment was relatively short. Further research is recommended to evaluate whey protein efficacy and safety, which might add more burden on the kidneys in the long term. Therefore, several doses of whey protein must be investigated in order to find the safest and most effective dose to reduce the cisplatin-induced nephrotoxicity,

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