Role of 6-amino flavone in attenuating cisplatin induced neurotoxicity via inhibition of p-JNK signaling pathway in post-natal day-7 mice

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ABSTRACT

OBJECTIVE: To investigate the protective role of 6-amino flavone (6-AF) in cisplatin-induced neuro-inflammation in the developing brains of post-natal day-7 (PND-7) mice.

METHODS: This experimental study, conducted at the Neuro Molecular Medicines Research Center in Peshawar, Pakistan, included 20 PND-7 mice from January to March 2023. PND-7 mice were randomly distributed into four groups, a control group, a cisplatin group, a cisplatin + 6-AF group and a 6-AF group. Cisplatin was administered intraperitoneally at a dose of 20 mg/kg to the cisplatin group. 6-AF was injected at a dose of 30 mg/kg after cisplatin administration to cisplatin + 6-AF group and 6-AF group mice. After 4 hours of the drug treatment, all the PND-7 mice were sacrificed for Western blot analysis. ImageJ software was used for the densitometry of the blots. One-way ANOVA and post-hoc Tukey tests through Prism Graph-5 were applied for statistical analysis.

RESULTS: Significant differences in p-JNK levels along with TNF-α, NF-κB, and IL-1β proteins were observed in the brain homogenates of PND-7 mice in various groups. A post-hoc Tukey test revealed a significant increase (p<0.001) in the p-JNK, COX-2, NF-κB, and IL-1β levels in the cisplatin group as compared to control group mice. However, a significant decrease (p<0.001) was observed in p-JNK, COX-2, NF-κB, and IL-1β expression levels in the cisplatin + 6-AF group as compared to the cisplatin group.

CONCLUSION: Administration of 6-AF effectively reduced cisplatin-induced neurotoxicity in PND-7 mice, demonstrating a neuroprotective effect by suppressing p-JNK and its downstream TNF-α, NF-κB, and IL-1β proteins.

KEYWORDS: Cisplatin (MeSH); 6-Amino flavone (Non-MeSH); Neuroinflammatory Diseases (MeSH); P-JNK signaling pathway (Non MeSH); NF-κB (Non-MeSH); Tumor Necrosis Factor-alpha (MeSH).

INTRODUCTION

Cisplatin, or cis-diaminedichloroplatinum (II), discovered in the mid-19th century by Michel Peyrone, emerged as a significant chemotherapeutic agent after its biological effects piqued scientific interest in the late 1960s. By 1971, cisplatin was undergoing clinical trials for cancer therapy, marking its introduction into the realm of cancer treatment. Despite the advent of numerous chemotherapy drugs over the years, cisplatin retains its status as a cornerstone in treating various human malignancies, including sarcomas, carcinomas, lymphomas, and germ cell tumors. The therapeutic use of cisplatin is not without adverse effects. Common short-term side effects encompass nausea and vomiting, experienced by virtually all patients undergoing treatment. More severe, dose-dependent side effects include ototoxicity, renal toxicity, hematologic disorders, gastrointestinal issues, and notably, neurotoxicity, manifesting as symptoms ranging from tingling and numbness to loss of proprioception and spinocerebellar ataxia. Notably, cisplatin-induced neurotoxicity represents a major dose-limiting factor, underlining the need for therapeutic strategies to mitigate this challenge. The oxidative stress induced by high doses of cisplatin contributes significantly to its toxicity profile. Studies in rat brain tissue have demonstrated an increase in pro-oxidants like lipid peroxidation and myeloperoxidase activity, alongside a reduction in antioxidants such as glutathione, highlighting the oxidative damage mechanism. The FDA’s approval of cisplatin in 1978 underscored its efficacy, making it one of the most potent drugs in the oncological arsenal. At the molecular level, cisplatin exerts its anti-cancer effects by crosslinking DNA’s purine bases, thereby disrupting DNA repair mechanisms and inducing apoptosis in cancer cells. In contrast to cisplatin’s toxicological profile, flavonoids—a diverse group of phytonutrients found in fruits, vegetables, flowers, wine, chocolate, and tea, encompassing approximately 9000 identified members—demonstrate antioxidant, anti-inflammatory, antimicrobial, and cardioprotective properties. These compounds, including subclasses like flavones, isoflavones, flavanols,
flavanonols, and flavanones, have gained attention for their therapeutic potential, particularly in formulations designed to leverage their antioxidant and anti-inflammatory effects.\(^1\)

Amid this backdrop, the synthetic flavone derivative, 6-aminoflavone (6-AF), exhibits pronounced anti-proliferative activity against MCF-7 breast tumor cells, signaling its potential as a chemotherapeutic agent.\(^2\) This study was planned to examine if 6-AF, a derivative of flavonoids, possesses antioxidant and anti-inflammatory capabilities comparable to those observed against potential neuro-inflammation caused by cisplatin in PND-7 mice, investigating its therapeutic efficacy.

**METHODS**

Healthy adult male and female albino mice (7-8 weeks old and 32 g body weight) were purchased from the Veterinary Research Institute, Peshawar, Pakistan. Mice were randomly distributed in 5 pairs. They were acclimatized to the environment and were provided with food and water ad libitum and a 12-hour light/dark cycle at 25 ±°C. The mice reproduced, and their pups were kept until the 7\(^{th}\) postnatal day. A resource equation approach was used for sample size calculation. The sample size was comprised of 20 PND-7 mice. Mice were randomly distributed into four groups, i.e.; a control group (n = 5), a cisplatin group (n = 5), a cisplatin + 6-AF group (n = 5), and a 6-AF group (n = 5). These mice were labeled and kept in separate cages (Biobase China) at the Neuro Molecular Medicines Research Center (NMMRC), Peshawar. A single dose of 20 mg/kg of cisplatin was administered intraperitoneally (IP) to cisplatin group PND-7 mice. On the

![Figure 1: 6-AF successfully ameliorated the expression level of p-JNK protein in the PND-7 mice brains of the cisplatin +6-AF group (shown are the Western blot results of p-JNK expression levels, along with their bar-chart representation in various experimental mouse groups, respectively). β-Actin was used as a loading control. The results were determined using Image J software, and the bar chart indicates the mean in A.U. ± SEM. The significance of a one-way ANOVA is expressed as α, the significance of control vs. cisplatin is expressed as #, and the significance of cisplatin vs. cisplatin + 6-AF is expressed as *. Significance: *** α, ** p<0.001).](image1)

![Figure 2: 6-AF demonstrated substantial amelioration in the expression level of TNF-α protein in the PND-7 mice brains of the cisplatin +6-AF group (shown are the Western blot results of TNF-α expression level, along with their bar-chart representation in various experimental mouse groups, respectively). β-Actin was used as a loading control. The results were determined using Image J software, and the bar chart indicates the mean in A.U. ± SEM. The significance of one-way ANOVA is expressed as α, the significance of control vs. cisplatin is expressed as #, and the significance of cisplatin vs. cisplatin +6-AF is expressed as *. Significance: *** α, ** p<0.001).](image2)
other hand, 6-mino-flavone was injected at a dose of 30 mg/kg after cisplatin administration to cisplatin + 6-AF group and 6-AF group mice. After 4 hours of cisplatin and 6-AF administration, all the PND-7 mice were sacrificed for laboratory investigations. It was an experimental study performed at NMMRC, Peshawar, Pakistan, from January 2023 to March 2023. Study was approved by the NMMRC animal ethical committee via Ref. No. 11/2022, dated: 05/03/2023.

All animals were sacrificed in accordance with the method described earlier. After decapitating the animals, we carefully and promptly removed the brain tissue and stored it on ice in a 1:1 mixture of RNA later solution and phosphate buffer solution (PBS). A total protein extraction solution (T-PER) was used to properly homogenize the brain tissue, which was then collected and kept at -20ºC for future analysis. Absorption at 595 nm was used to measure protein concentration in homogenates using a Bio-Rad protein estimation assay. SDS-PAGE at a concentration of 12–15 percent was used to electrophorese each sample's protein after it had been standardized to 30mg and 50 mA was used for around 20–30 minutes, then 120 V was used for about 1–1.5 hours till the run was finished. Semi-dry Bio-Rad Transblot technology was used to transfer proteins from gel to polyvinylidene difluoride (PVDF) membranes (Santa Cruz, CA, USA). Various mouse-derived primary antibodies, such as anti-NF-kB (SC-8414), anti-TNF-a (SC-52746), anti-IL-1β (SC-12742), and anti-p-JNK (phospho-c-Jun N-terminal kinase) (SC-6254) manufactured by Santa Cruz Technologies, USA, were employed, while HRP-conjugated secondary antibodies were procured from Promega, Melbourne, USA. The trans-blot results of electrophoresis were developed on X-ray films using chemiluminescence assay reagents.

The original X-ray films of the Western blot analysis were scanned. Image J software was used to perform densitometry analysis of the bands. Integral optical density (IOD) of proteins was expressed in arbitrary units (A. Us) as mean±S.E.M. A significant difference was determined using one-way analysis of variance (ANOVA), followed by a post-hoc Tukey’s test on GraphPad Prism 5. A p-value less than 0.05 (P ≤ 0.05) was considered statistically significant.

RESULTS
p-JNK is known as a stress-activated protein kinase. Many stressful conditions cause overexpression of the p-JNK protein, which is actively involved in the mediation of inflammatory and apoptotic signaling. Cisplatin is the most common cause of the activation of phospho-JNK proteins. Therefore, we analyzed the p-JNK level through Western blotting. One-way ANOVA analysis showed significant differences in p-JNK levels in various groups. A post hoc Tukey test revealed that cisplatin caused a significant increase (p<0.001) in the phospho-JNK level in the adult mouse brain. However, 6-AF significantly rectified (p<0.001) phospho-JNK in cisplatin + 6-AF group (Figure 1).

One-way analysis showed significant differences (p<0.001) in the expression levels of all the assessed neuroinflammatory markers, i.e., COX-2, IL-1β, and NF-kB, in the brain homogenates of mice of various groups. A post-hoc Tukey test revealed that cisplatin significantly increased (p<0.001) COX-2, IL-1β, and NF-kB levels in mice brains. However, 6-HF administration significantly inhibited (p<0.001) the expression of COX-2 (Figure 2), IL-1β (Figure 3), and NF-kB proteins (Figure 4) in the cisplatin + 6-AF group mice as compared to the cisplatin group. These findings ascertained 6-AFs role as an anti-neuroinflammatory agent.

DISCUSSION
Our study demonstrated that 6-AF effectively mitigates cisplatin-induced neuro-inflammation in PND-7 mice. Analyses of brain homogenates revealed a significant increase (p<0.001) in p-JNK, COX-2, NF-kB, and IL-1β in cisplatin-treated mice compared to the control group. Conversely, cisplatin + 6-AF treatment resulted in a significant decrease (p<0.001) in the expression of these markers compared to the cisplatin-only group.

An increased inflammatory response has been associated with the development of cisplatin-induced neuro-inflammation. Many studies have reported that pro-inflammatory cytokine induction contributes to the pathogenesis and exacerbation of nervous tissue damage in cisplatin administered animals. To reduce inflammation in cisplatin-induced neurotoxicity, several in vivo approaches have been recommended, but it is unclear whether such approaches limit the anticancer efficacy of cisplatin.

C-Jun N-terminal protein kinase (JNK) belongs to mitogen activated protein kinase (MAPK) superfamily. JNK can specifically phosphorylate the transcription factor c-Jun on its N-terminal transactivation domain at two serine residues, Ser63 and Ser73. Subsequent studies revealed that JNK also phosphorylates and regulates the activity of transcription factors and non-transcription factors, such as neuroinflammatory and neurodegenerative factors, in response to a variety of extracellular stimuli.

Inflammatory disease therapies specifically target the JNK pathway. The synthesis of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, along with the maturation and activity of T cells, are regulated by JNK. Several recent studies have established the importance of the JNK pathway in chronic inflammatory disorders involving the expression of specific proteases and cytokines. Depending on the stimulus and cell type, JNKs can phosphorylate several members of neuro-inflammation and apoptosis-related proteins.

Multiple studies have demonstrated that pro-inflammatory cytokine induction, together with macrophage and neutrophil infiltration, contributes to the pathogenesis and exacerbation of many diseases in cisplatin-administered animals. Hence, inflammatory reaction modulation may help to prevent cisplatin-induced neuro-inflammation. Although different
treatment strategies have been proposed to decrease cisplatin-induced inflammation, it is not clear whether such treatment strategies alter the anticancer efficacy of cisplatin.

Several cytokines (e.g., TNF-α, IL-1β, MCP-1, and IL-6) are upregulated in the inflammatory cascade triggered by cisplatin. TNF-α plays a key role in cisplatin-induced neuro-inflammation by activating a variety of signaling pathways with cellular responses ranging from the activation of inflammation to cell death. Strong evidence affirm the vital role of NF-κB activation in the pathogenesis of cisplatin-induced neuro-inflammation. NF-κB regulates neutrophil, macrophage, and lymphocyte biology, as well as the synthesis of hundreds of proinflammatory genes, including TNF-α, thus amplifying the process of inflammation.

A study reported that the liver and kidney contents of the inflammatory mediators TNF-α and NF-κB are increased along with the infiltration of neutrophils by cisplatin. The crucial pro-inflammatory mediator TNF-α is released by macrophages activated by neutrophils. TNF-α, then, by binding to its receptor, promotes the release of NF-κB. Furthermore, the subsequent release of NF-κB and its translocation into the nucleus activate the transcription of multiple genes, including IL-1β and TNF-α. These findings are very consistent with our findings. Our study revealed that cisplatin administration induced increased activation of the p-JNK protein along with the significant increase in its downstream inflammatory mediators TNF-α, NFκB and IL-1β.

Flavonoids are phenolic compounds that are currently under major attention in the field of drug discovery. Researchers used kaempferol, a naturally occurring flavonoid, against cisplatin-induced nephrotoxicity by...
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modulating oxidative stress, inflammation, and apoptosis via the ERK and NF-kB pathways. They observed that, Kaempferol pre-treatment decreased the release of pro-inflammatory cytokines (IL-12 and TNF-α) and also modulated the levels of NF-kB, thereby ameliorating the cisplatin mediated inflammation.25 Lu L et al., (2023) utilized the flavonoid derivative DMXAA to mitigate cisplatin-induced acute kidney injury.26 Altimdag F et al., (2022) employed silymarin to address cisplatin-induced nephrotoxicity in Albino Wistar male rats, revealing reduced TNF-α and NF-kB expressions and increased IL-10 expression with silymarin treatment.27 Alsayari et al., (2019) demonstrated that Aurone, a benzofuranone, targets various mechanisms in cancer cells because of many possible targets, including cyclin-dependent kinase, telomerase, histone deacetylase, sirtuins, adenosine receptor, and microtubules.28 Fisetin, another flavonoid, exhibits antioxidant and anti-inflammatory effects,29 while flavonols from cinnamon activate Nrf2, combating oxidative stress.30

In this study, we demonstrated for the first time that 6-AF mitigates cisplatin-induced neuro-inflammation in PND 7 mice brains by inhibiting p-JNK activation and reducing key pro-inflammatory cytokines such as TNF-α, NF-kB, and IL-1β. Our findings align with previous research, showing a significant increase in these inflammatory markers in cisplatin-treated PND 7 mice brains. Treatment with 6-AF notably decreased these proinflammatory markers, thus alleviating inflammatory damage. The mechanism underlying this protection—whether causal or consequential—remains to be clarified. Our results suggest that 6-AF has potential as an adjunctive therapy in cancer treatment, offering protection against cisplatin-induced neuro-inflammation.

**CONCLUSION**

In conclusion, 6-amino flavone decreased cisplatin-induced neurotoxicity in the PND-7 mice brain. The neuroprotective effect observed was associated with suppression of p-JNK protein along with its downstream TNF-α, NF-kB, and IL-1β proteins in the brain homogenates of PND 7 mice. Hence, we got strong evidence from a recent study that 6-amino flavone has higher efficacy against cisplatin-induced neuro-inflammation.

**RECOMMENDATION**

Future studies are also needed to explore impact of different dose of these drugs (6-amino flavone) by which above cisplatin induced neuro-inflammation can be reduced.

**REFERENCES**

019-0518-9
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CONFLICT OF INTEREST
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