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The potential of Roflumilast and Melatonin on nephrotoxicity induced by Cisplatin by oxidative stress regulation

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ABSTRACT

Background and Aim: Cisplatin (Cis) is a very effective anticancer medication that is utilized to treat a diverse range of cancers. Nevertheless, the application of Cis is restricted due to its significant adverse effects, such as nephrotoxicity. Antioxidant therapies partially mitigated the oxidative damage generated by cisplatin in the kidney. Melatonin and Roflumilast have antioxidant properties via scavenging free radicals. Hence, we conducted this work to examine the underlying mechanism by which ROF and Mel protect against Cis-induced kidney injury. **Materials and Methods**: Fifty male Sprague-Dawley rats were divided into five groups. Control group: Animals were given a 0.9% saline solution. Experimental group: The rats were administered a single dosage of 6 mg/kg. ROF group: The rats were administered a dose of 1.2 mg/kg. The Mel group was administered a dose of 10 mg/kg/day. The ROF + Mel group had a Cis injection before being delivered. Urine and blood samples were gathered on day 5 and day 11 to undergo chemical analysis, while kidney samples were acquired for molecular and biochemical investigations.

Results: The concentrations of blood creatinine, BUN, and total protein were higher in the Cis group compared to the control group ($p < 0.05$). Nevertheless, the joint effect of ROF and Mel substantially reduced these values at the 5-day and 11-day marks ($p < 0.05$). Furthermore, Cis induced renal oxidative stress by increasing MDA levels and inhibiting the functions of SOD, GSH, and CAT. Furthermore, the influence of ROF and Mel on these effects was observed after 11 days ($p < 0.05$). In addition, the simultaneous administration of ROF and Mel led to a notable upregulation of Nrf2 and HO-1 expression compared to the Cis group after 11 days ($P < 0.05$).

Conclusion: The study shown that the kidneys can be preserved against Cis-induced injury by combining cisplatin-based medications with ROF and Mel antioxidant-based therapy interventions. These interventions elevate antioxidant levels, hence reducing the detrimental effects of reactive oxygen species (ROS) damage. These therapeutic methods can improve the body's ability to tolerate Cis, allowing for higher doses and leading to improved results.

Introduction

Cisplatin (Cis) and its platinum analogues are very effective chemotherapeutic agents utilized in the treatment of diverse malignant tumors, such as ovarian, bladder, cervical, lung,

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testicular, and head and neck cancers[1]. Cis induces cytotoxicity by creating covalent linkages with nucleophilic purine-N7 sites in DNA, hindering the processes of DNA transcription and replication, and ultimately leading to cell death [2]. Despite its potency as a chemotherapeutic drug, the use of Cis is restricted due to notable adverse effects, including organ damage. Cis induces cardiotoxicity, neuropathy, ototoxicity, nephrotoxicity, and hepatotoxicity [3-6].

Acute kidney injury (AKI) is a significant negative consequence of Cis treatment, since Cis builds up in the kidney and leads to a decrease in glomerular, vascular, and tubular functions [3-6]. Cis -induced nephrotoxicity is caused by several processes, including reactive nitrogen species, reactive oxidative stress (ROS), inflammation, apoptosis, necrosis, fibrogenesis, and hypoxia [7].

Extensive research has been conducted to find various preventative methods for effectively controlling the nephrotoxicity caused by Cis. However, there is a limited number of therapy options that have been proposed and implemented in clinical practice for treating Cis-induced nephrotoxicity. Currently, the practice of hydrating with magnesium supplements and mannitol is widespread, but there is ongoing debate over its usefulness [8]. Cystone gives protective effects in nephrotoxicity induced by Cis [9]. Currently, there is no effective and definitive treatment available to prevent Cis-induced kidney damage. However, the use of anti-inflammatory and antioxidant agents has emerged as the main strategy to develop therapies that can inhibit or minimize Cis-induced kidney damage.

Roflumilast (ROF) is a powerful and specific inhibitor of phosphodiesterase-4 (PDE4), used to treat and reduce the likelihood of exacerbations in chronic obstructive pulmonary disease (COPD), particularly in patients with severe COPD associated with chronic bronchitis. Targeted suppression of PDE4 hampers the breakdown of cyclic adenosine monophosphate (cAMP) in inflammatory cells. Elevated intracellular cAMP leads to various anti-inflammatory outcomes, such as diminished release of inflammatory mediators in neutrophils, declined cytokine release, diminished expression of cell surface markers in multiple cell types, and decreased apoptosis [10].

The pineal gland synthesizes and releases melatonin (Mel) into the circulation, particularly into the cerebrospinal fluid [11]. Also, Mel is produced by other organs like brain, immune system cells, airway epithelium, gut, bone marrow, testes, ovary, and skin [12] . Mel and its metabolites have antioxidant properties via scavenging free radicals [13]. Mel exhibits both receptor-independent and receptor-mediated mechanisms and is thought to impact all types of cells [14]. Mel enhances the expression of antioxidant enzymes at both the mRNA and protein levels via activating Nrf2 [15]. Increasing the level of Nrf2 by Mel lead to an upregulation in the expression of antioxidant enzyme heme oxygenase-1 (HO-1) [16].

Studies have demonstrated that ROF and Mel have a protective effect against Cis toxicity [17, 18]. Nevertheless, the precise mechanism by which they confer protection against Cis -induced nephrotoxicity remains unclear. Consequently, we conducted this investigation to examine the mechanism behind the synergetic effect of ROF and Mel in protecting against Cis-induced kidney injury.

MATERIALS AND METHODS:

Chemicals

Cisplatin (Cis), Melatonin (N-acetyl-5-methoxytriptamine) (Mel) and Roflumilast (ROF) were purchased from Sigma-Aldrich.

Experimental Animals

A total of fifty male Sprague Dawley (SD) rats, aged 8 weeks and weighing between 200 and 215 grams, were acquired. The rats were housed in a room with controlled environmental conditions, maintaining a constant temperature of 21 ± 1°C and humidity of 75 ± 5%. The rats were subjected to a 12-hour light/dark cycle. The rats were adapted for one week before the study and were provided with unrestricted access to water/ ad libitum. The project has obtained approval from the Ethics Review Committee for Ethics in Animal Experiments at the Faculty of Science, Zagazig University. (Approval number ZU-IACUC/1/F/132/2021). The guidelines for the care and use of laboratory animals were adhered to very rigorously.

Experimental design

Male SD rats (n = 50) were randomly distributed into five equal groups, as described in (Table 1). Animals were scarified at 5 and 11 days (n = 5/group) in each time. Urine, blood, and tissue samples were taken at each time point.

Laboratory tests

A Detection of kidney function included serum creatinine and blood urea nitrogen (BUN) was achieved using Architect c4000 system. The total urinary protein was detected using Fortress Diagnostics Limited Unit 2C Antrim Technology Park kit (cat .no. Antrim BT41 1QS, United Kingdom) [19].

Detection of oxidative stress and antioxidant markers

About 100 mg of kidney tissues were homogenized in pH 7.4, 50 mM cold phosphate buffer saline to detect the levels of malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) using a colorimetric method. The used kits were provided by Biodiagnostic (Giza, Egypt) [20, 21].

Gene expression assay

m-RNA was extracted from renal tissues by TRIZOL reagent (Thermo Scientific, USA). The concentration and purity of m-RNA were assessed using the Nano-Drop 2000c spectrophotometer (Thermo Scientific, USA). Reverse transcription kit (Applied Biosystems, USA) was used to convert the m-RNA into cDNA. The NRF2 and Ho-1 sequences are mentioned in (Table 2). The gene expression was normalized using the housekeeping gene (GAPDH). The gene expression was detected according to equation $2^{-\Delta\Delta ct}$[22].

Statistical analysis

The results were expressed as mean ± SD. One-way ANOVA and the post hoc test were used to compare groups using SPSS software (IBM Corporation). P-value < 0.05 was considered significant. The graphs were drawn using Prism 8 GraphPad.

RESULTS:

Kidney function

Renal levels of serum creatinine (SCr), blood urea nitrogen (BUN), and total protein were assessed after 5 and 11 days of Cisplatin administration (Table 3). Compared to the control group, Cis group showed high levels of SCr, BUN, and total protein after 5 and 11 days ($p < 0.05$). Treatment with Mel showed a significant decrease in BUN compared to Cis group after 5 and 11 days ($p < 0.05$), while levels of Scr and T. protein only decreased after 11 days ($p < 0.05$). Also, groups treated with ROF showed a significant reduction in SCr, BUN, and T.protein levels compared to Cis group after 11 days ($p < 0.05$). Moreover, treatment with both Mel and ROF

revealed the most reduction in SCr, BUN, and T.protein levels compared to Cis, Mel, and ROF groups after 11 days($p < 0.05$).

Oxidative stress assays:

The levels of SOD, CAT, GSH, and MDA were measured in the various treated groups after 5 and 11 days of treatment (Figure 1). The activity of GSH, CAT, and SOD was significantly decreased with the induction of Cis compared to the control group after 5 and 11 days ($p < 0.05$). Groups treated with either Mel or ROF showed a significant increase in GSH, CAT, and SOD compared to Cis group after 5 and 11 days ($p < 0.05$), while group treated with both Mel and ROF showed the best activity of GSH, CAT, and SOD compared to Cis, Mel, and ROF groups at 11 days ($p < 0.05$). On the other hand, Cis group showed a high concentration of MDA compared to the control group ($p < 0.05$), whereas treatment with Mel or ROF reduced MDA level ($p <0.05$). Furthermore, treatment with the combination Mel and ROF manifested the best reduction in MDA levels among the other treated groups at 11 days ($p < 0.05$).

Gene expression analysis:

The levels of antioxidant genes Nrf2 and HO-1 were compared between groups after 5 and 11 days (Figure 2). The gene expression of Nrf2 and HO-1 was significantly low in Cis group compared to the control group at 5 and 11 days ($p<0.05$), while their expression was raised by treatment with Mel or ROF ($p<0.05$). Moreover, the administration of both Mel and ROF showed the most increase in the expression of Nrf2 and HO-1 compared to groups treated with Cis, Mel, and ROF after 5 and 11 days ($p<0.05$).

DISCUSSION:

Despite being widely used in clinical practice, cisplatin causes significant nephrotoxicity. Hence, there is a pressing clinical requirement for approaches to alleviate, if not entirely prevent, the renal toxicity caused by this medication. The present strategies, such as dehydration, are only partially effective. Therefore, there is a need to find safe and effective methods of co-administering appropriate medicines that allow for the delivery of high doses of Cis safely. While some phytochemicals have shown promising outcomes in animal research for improving Cis nephrotoxicity, none of them have been evaluated on human patients [23]

Oxidative stress is a significant contributor to the development of acute kidney injury (AKI) caused by cisplatin. Oxidative stress leads to a rise in reactive oxygen species (ROS) [24]. Multiple investigations have demonstrated that antioxidants exert a safeguarding influence on cisplatin-induced nephrotoxicity via modulating inflammation and oxidative stress [25]. We have confirmed that the administration of ROF and melatonin, individually, effectively alleviated almost all the physiological, biochemical, and molecular changes induced by Cis in laboratory rats.

Previous research has firmly established that administering a single injection of cisplatin at a dosage of 6 mg/kg body weight to mice results in evident damage to the kidneys, as indicated by elevated levels of urea nitrogen (BUN) and serum creatinine (SCr), along with noticeable alterations in the structure of kidney tubules [26]. The present investigation has verified the toxic impact of cisplatin on the kidney. This is evident from the reduced renal function observed in the Cis group, as evidenced by a significant elevation in serum creatinine, blood urea nitrogen (BUN),

and total protein levels compared to both the control and treated groups. The results of this study align with prior research [27, 28] and indicate a decrease in the filtration of creatinine and BUN, presumably due to damage to the renal blood vessels caused by cisplatin. This leads to constriction of the renal blood vessels, reduced blood flow to the kidneys, and ischemia injury [29].

Melatonin, once recognized solely as the "sleeping hormone," has now been demonstrated to contain antioxidant characteristics. Melatonin effectively reduced serum creatinine and urea levels compared to cisplatin. This finding aligns with the studies conducted by Ko et al. (2019), Ali et al. (2020), and Elsamman et al. (2024), which attributed the improvement to the antioxidant, anti-inflammatory, and anti-apoptotic properties of melatonin [23, 30, 31].

Additionally, there have been reports indicating that Roflumilast (ROF), which is a substance that inhibits the PDE4 enzyme, has the ability to protect the nephrons and reinstate their regular functioning. Additionally, it possesses antioxidant characteristics that can replenish the levels of renal function indicators in animal models of nephrotoxicity [32]. Administration of ROF significantly reversed the nephrotoxic effects caused by Cis, as seen by the decrease in levels of creatinine, BUN, and total protein. This study confirms previous research that has demonstrated the role of ROF in the restoration of renal function [32]. Moreover, the simultaneous administration of ROF and Mel demonstrated the most favorable levels of creatinine, BUN, and total protein. Proposing the dual impact of utilizing a combination of antioxidant agents on AKI.

Oxidative stress can be detected through the presence of specific biomarkers, including reactive oxygen species, nitrogen species, lipid peroxidation, and several antioxidant enzymes. Oxidative stress is a key factor in the development of kidney damage caused by Cis. Previous studies have indicated that the administration of Cis results in an excessive formation of free radicals, specifically hydroxyl radicals and superoxide anions, which subsequently leads to oxidative damage and lipid peroxidation in the kidneys. Furthermore, it was demonstrated that Cis causes harm to antioxidant defense mechanisms, resulting in a decrease in SOD levels and an increase in MDA content [33]. This study revealed that rats treated with Cis showed reduced activity of antioxidant enzymes in their kidneys. These enzymes are metalloproteins that remove harmful peroxides (–OOH), H2O2, and O2.- from the body. The structure and function of these antioxidant enzymes rely on important trace elements and prosthetic groups. As a result, they are vulnerable to the cytotoxic effects of cisplatin and can be targeted by it [34]. Hence, the reduced functioning of the antioxidant enzymes GSH, SOD, and CAT found in rats treated with Cis signifies a deficiency in the antioxidant defense system's ability to counteract the increase in ROS caused by exposure to cisplatin.

Studies have shown that Mel has been observed to remove reactive oxygen and nitrogen species and activate antioxidant enzymes such as SOD and GR [35]. Melatonin can directly neutralize a range of harmful free radicals, including O, OH, ONOO, and H2O2. In aerobes, oxidative stress is primarily caused by the reduction of molecular or ground state oxygen into a highly reactive free radical called superoxide anion (O2−). This free radical can then interact with other molecules and change into even more harmful free radicals like as OH, ONOO, and H2O2. Antioxidants naturally scavenge the superoxide anion (O2−), converting it into H2O2, which is then metabolized into water and oxygen [36].

Furthermore, our investigation unveiled that the adoption of the ROF resulted in a significant increase in the GSH, CAT, and SOD levels and a decrease in the MDA level. The results are consistent with the study conducted by Ansari et al. (2019), which demonstrated that ROF had protective effects against Cadmium-induced kidney damage and led to a significant increase in GSH levels [32]. Administering Cis exposure during ROF treatment successfully reduced oxidative stress and restored CAT and SOD activity in kidney tissues, providing a considerable protective effect. We further investigated whether the combined administration of ROF and Mel may provide enhanced mitigation of Cis-induced nephrotoxicity in treated rats, while avoiding any negative side effects. The combination of ROF-Mel enhanced all the quantified indicators of oxidative stress, it enhanced the functioning of antioxidant enzymes, specifically GSH, CAT, and SOD.

The production of ROS by Cis is required for the activation of antioxidant response elements (ARE) through the Nrf2-driven transcriptional process. As a result, we anticipated that Cis might cause the movement of Nrf2 into the nucleus and trigger the activation of NF-κB. The activation of NF-κB by ROS has been documented in a prior investigation [37]. Nrf2 is a fundamental leucine zipper transcription factor that controls the transcription of several genes, such as HO-1, NAD(P) H:quinine oxidoreductase-1, c-glutamylcysteine synthase, and glutathione S-transferase [38]. Nrf2 shields the cell against oxidative stress by stimulating the expression of various phase 2 detoxifying and antioxidant enzymes, with a specific emphasis on HO-1 [39], through the activation of ARE. HO-1 is an enzyme that responds to stress and is responsible for breaking down heme into biliverdin, free iron, and carbon monoxide [40]. It is triggered by several cellular stressors, such as heme, excessive oxygen, lack of oxygen, and electrophiles [39]. According to Beni et al. (2004), the activation of the transcription factor Nrf2 is affected by the cellular redox state, which serves as a detector of electrophiles and prooxidant stresses [41].

In the present study, the group treated with Mel demonstrated a noteworthy augmentation in the expression of Nrf2 and HO-1. This finding corresponds to previous research that indicated Mel's ability to enhance indicators of oxidative stress by increasing the expression of the antioxidant and detoxification enzyme HO-1 [42].

In addition, the ROF therapy showed a significant increase in the expression of Nrf2 and HO-1. Our study corroborates the findings of Patel et al. (2023), who noted that ROF can enhance the expression of the Nrf2 gene in a diabetic nephropathy model. These findings indicate that ROF may have the potential to act as a nephroprotective agent [43]. A study conducted by Abdel-Wahab et al. found that an increase in the levels of Nrf2, HO-1, and NQO-1 occurred when the cAMP level increased. This increase was due to the inhibitory impact of ROF on PDE4 during testicular toxicity by Cis. These findings indicate that the upregulation of HO-1 and NQO-1, induced by ROF, results in an elevation in the production of both non-enzymatic and enzymatic antioxidant components [18]. The group that received both ROF and Mel exhibited the most significant increase in Nrf2 and HO-1 expression, suggesting that their combined use had a strong protective effect as very potent antioxidant agents.

The combination of ROF and Mel did not result in any apparent interactions that caused obvious toxicity, detrimental effects on the animals, or reduced the effectiveness of either substance. However, additional research is necessary to investigate the safety and efficacy of various doses of a

combination of ROF and Mel in experimental animals. Until additional pharmacological and toxicological research is conducted on this combination, we suggest that a limited number of patients receiving Cis should be tested to determine whether this combination would maintain Cis's effectiveness while significantly reducing its nephrotoxicity. Furthermore, recent results indicate that melatonin may possess potential anticancer benefits when administered alone [44]. If clinical confirmation is obtained, it will provide additional credibility for the concurrent administration of the pair with cisplatin.

CONCLUSION:

Our findings shown that the kidneys can be preserved against Cis-induced injury by combining cisplatin-based medications with ROF and Mel antioxidant-based therapy interventions. These interventions elevate antioxidant levels, hence reducing the detrimental effects of reactive oxygen species (ROS) damage. These therapeutic methods can improve the body's ability to tolerate Cis, allowing for higher doses and leading to improved results.

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Table 1: Experimental design:

Group no.	Group name	Treatment
1	Control group	Animals were injected with 0.9 % saline intraperitoneal (i.p.).
2	Cis group	Rats were injected i.p. with single dose of Cis (6 mg/kg) [45].
3	Mel group	Rats were received 10 mg/kg/day after Cis injection [46].
4	ROF group	Rats were administered with roflumilast orally (1.2 mg/kg), after Cis injection [47].
5	Mel+ROF group	rats received 10 mg/kg/day of Mel and 1.2 mg/kg of ROF after Cis injection.

Table 2: list of primers sequence:

Primer	Accession Number	Sequence
GAPDH	NM_017008.4	F:TATCGGACGCCTGGTTAC R:CTGTGCCGTTGAACTTGC
NRF-2	NM_001399173.1	F: GCTATTTTCCATTCCCGAGTTAC R: ATTGCTGTCCATCTCTGTCAG
HO-1	NM_012580.2	F: CTTTCAGAAGGGTCAGGT GTC R: TGCTTGTTTCGCTCTATCTCC

Table 3: The levels of Serum creatinine, BUN, and Urine total protein between the different groups.

	SCr (mg/dL)		BUN (mg/dL)		T. protein (mg/dL)	
Groups	5 days	11 days	5 days	11 days	5 days	11 days
Control	0.6±0.09	0.58±0.07	21.18±1.38	22.15±1.31	5.11±0.51	5.33±0.23
Cis	4.12±0.81[a]	5.11±0.23[a]	71.3±2.38[a]	72.03±2.91[a]	9.74±1.45[a]	10.6±0.47[a]
Cis+ROF	3.44±0.3[a]	1.5±0.28[ab]	58.73±1.84[a]	48.31±1.53[ab]	8.72±0.32[a]	8.43±0.25[ab]
Cis+ Mel	3.7±0.33[a]	1.75±0.22[ab]	61.15±2.31[ab]	52.41±2.13[ab]	9.12±1.16[a]	8.33±0.32[ab]
Cis+ROF+Mel	2.42±0.21[ab]	1.3±0.12[ab]	48.0±2.82[abd]	37.32±1.45[abcd]	7.1±0.79[abc]	6.22±0.21[abcd]

All data are expressed as mean ± SD. [a]Significant vs Control, [b]Significant vs Cis, [c]Significant vs Cis+ROF, and [d]Significant vs Cis+Mel. Statistical significance was determined where $P < 0.05$.

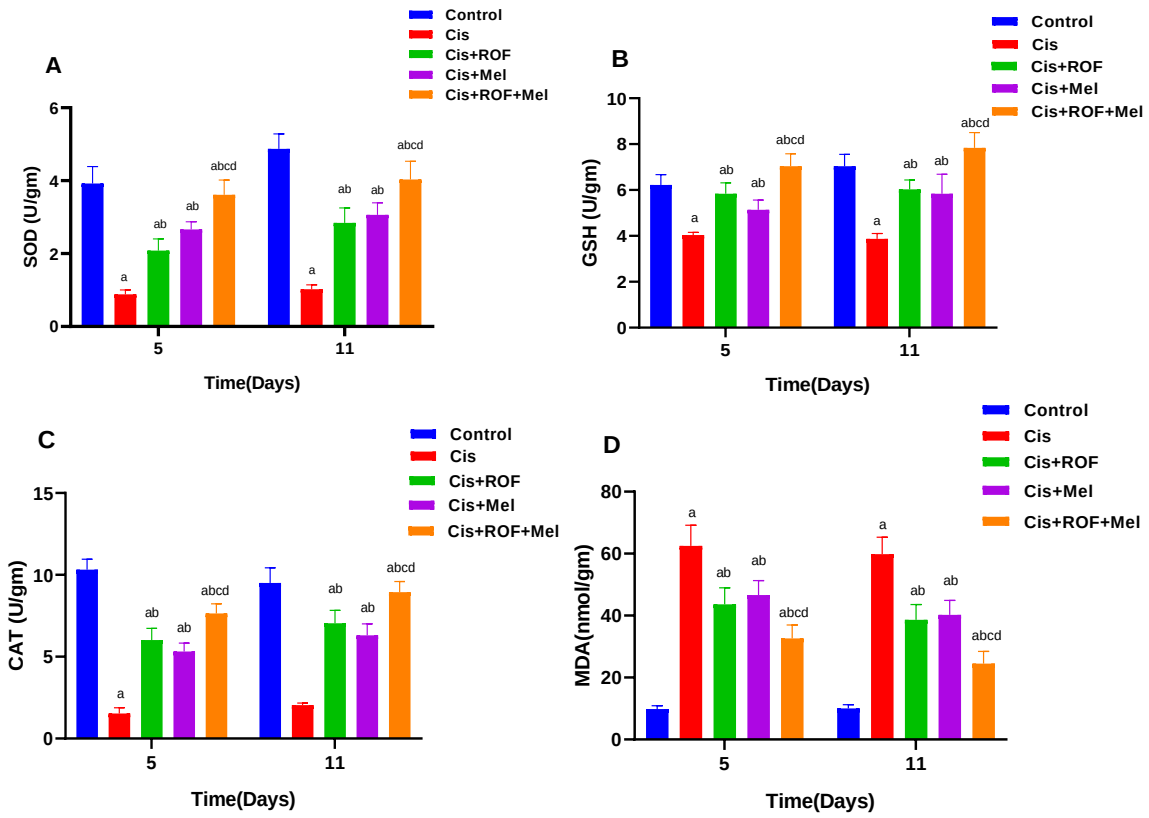

Figure (1): Effect of ROF and Mel on A) SOD, B) GSH, C) CAT, and D) MDA levels among the studied groups. [a]Significant vs Control, [b]Significant vs Cis, [c]Significant vs Cis+ROF, and [d]Significant vs Cis+Mel. Statistical significance was determined where *P* <0.05.

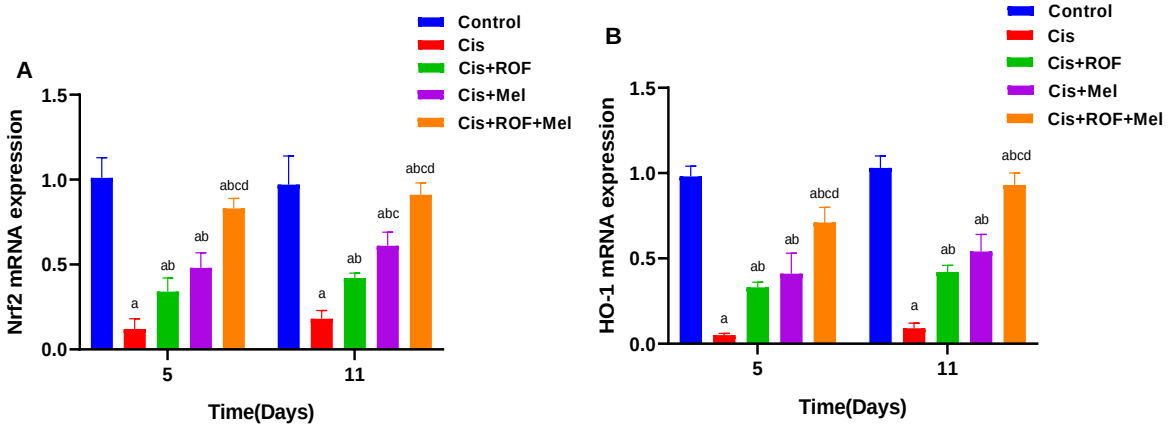

Figure (2): Gene expression for Nrf2 and HO-1 in the different treated groups. aSignificant vs Control, bSignificant vs Cis, cSignificant vs Cis+ROF, and dSignificant vs Cis+Mel. Statistical significance was determined where *P* <0.05.