



Metallothionein induction ameliorates cisplatin-induced hepatotoxicity: Molecular and Cellular Study

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General Note



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ABSTRACT

Cisplatin is effective as a chemotherapy treatment, but its toxicity is one of the undesirable effects that limit cisplatin use in cancer patients. The central hypothesis of this study was that cisplatin induces hepatotoxicity by increasing reactive oxygen species (ROS), thus induction of one of the most effective antioxidants metallothionein (MT) within the cells can ameliorate the cytotoxicity induced

by cisplatin. An *in vitro* model of hepatocytes, the cell line HepG2, were treated by different concentrations of cisplatin, which induced a dose dependent significant reduction in cell viability. HepG2 cells were treated with zinc sulphate to induce metallothionein. The MTT results showed enhanced cell viability in HepG2 cells pre-treated with zinc sulphate compared to cells treated with cisplatin alone. Reactive oxygen species (ROS) levels were quantified and resulted in that the HepG2 cells treated with cisplatin displayed a significant higher concentration of ROS than control cells, while cells preincubated with zinc followed by cisplatin show a significant reduction in ROS production. Gene expression data showed a significant increase in the expression of metallothionein 2A (MT2A) in HepG2 cells pretreated with zinc sulphate when compared to cells treated with cisplatin alone. Treating cells with zinc generally produced a protective effect against cisplatin-induced hepatotoxicity. The protective effect of zinc is associated with its antioxidant properties, as it acts as a metallothionein inducer.

Keywords: cisplatin, metallothionein, hepatotoxicity, ROS, drug toxicity, free radicals, MT2A, liver.

1. INTRODUCTION

Cisplatin is a commonly antineoplastic drug and is one of the most effective chemotherapies used to treat cancer (Cepeda et al., 2007). Despite the potent anticancer activity of cisplatin, the limitation in its clinical use is the undesirable toxicity, especially kidney toxicity, and at increasing doses, it can cause hepatotoxicity (Cavalli et al., 1978; Liu et al., 1998; Zicca et al., 2002). The mechanism of action of cisplatin is believed to result from intra- and inter- strand DNA cross-links, however the cytotoxicity that leads to apoptosis induction is believed to be resulted from the formation of cisplatin-DNA adducts that inhibit DNA replication (Eastman, 1990). Many potential reasons have been suggested to explain cisplatin hepatotoxicity, such as the accumulation in target organs via covalent binding with proteins intracellularly (Pera et al., 1987), though there is still discussion regarding cisplatin-induced liver toxicity.

Antioxidants are known to protect against drug-induced reactive oxygen species. One of those antioxidants is metallothionein (MT), which is a greatly inducible few molecular weight proteins which characterized, by high concentrations of heavy metals (Carpenè et al., 2007). It is composed of a 61 amino acid single-chain polypeptide and elevated concentration of cysteine residues with no disulphide bonds, instead these binds to copper or zinc ions in thiolate clusters. MT exists in mammals in 4 isoforms (MT-1 to MT-4), all of which have metal-binding locations; MT-1 and MT-2 are ubiquitously expressed and also MT-3 is found in the brain and MT-4 is found in squamous epithelia (Nielson & Winge, 1983; Sato et al., 1993). Some metals, including cadmium and zinc, work as stimuli and cause MT induction. Under oxidative conditions, the induction of MT has led to speculation that MTs may have a protective effect and can work as free radical scavengers, and this is confirmed by a considerable amount of data. MT-null mice were found to be more sensitive to cisplatin hepatotoxicity (Liu et al., 1998, Habeebu et al., 2000). This raises the question "would induction of MT protects liver from cisplatin toxicity?". Thus, the major target of the current study was to examine for molecular and cellular events in response to cisplatin hepatotoxicity by studying the essential role that (ROS) play in hepatotoxicity and confirm that oxidative stress is one of the main causes of cisplatin-induced hepatotoxicity. The current study will identify the molecular (MT gene expression) and cellular mechanisms (ROS production) that lead to cisplatin hepatotoxicity and answer the question "does oxidative stress has an important role in cisplatin-induced hepatotoxicity?". Finally, it will demonstrate if MT induction has a protective effect toward cisplatin-induced hepatotoxicity.

2. MATERIALS AND METHODS

Materials

The materials used were Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin and 0.25 % trypsin-ethylene diamine tetra acetic acid (EDTA) (UFC Biotech), Foetal bovine serum (FBS) (Gibco), Phosphate buffered saline (PBS) tablets (MP Biomedical), pure Dimethyl Sulfoxide (DMSO), (Thiazolyl Blue Tetrazolium Bromide) and Cisplatin (Sigma-Aldrich), zinc sulphate heptahydrate (Techno Pharmchem), Dichloro dihydro fluorescein Diacetate (2',7'-Dichlorofluorescein diacetate) (Invitrogen), and ROS-Glo™ H₂O₂ assay (Promega).

All laboratory work was carried out in the tissue culture unit, genome centre and biochemistry experimental unit in King Fahd Medical Research Centre (KFMRC).

Methods

HepG2 culture

The human hepatic cell line HepG2 was a kind gift from Dr. Etmad Huwait (Department of Biochemistry). HepG2 cells were routinely cultured in DMEM with phenol red containing glucose 4.5 g/L and L-glutamine 4 mM (Sigma-Aldrich), complemented with 10 % FBS, and 1 % penicillin-streptomycin antibiotics. Cells were kept at 37 °C in a humidified incubator with an atmosphere of 5 % CO₂. Cells were routinely sub-cultured when they were nearly 70-80 % confluent (every 2-3 days).

Treatment of Cells with Xenobiotics

HepG2 cells were seeded as required in tissue culture flasks or multi-well plates. After 24 h incubation, the culturing medium was aspirated off and the cells were washed with serum free medium. Cells were then treated with serum free medium containing either xenobiotic or vehicle control. Xenobiotics were prepared by being dissolved in a relevant solvent (DMSO). The stock was then diluted as needed in serum free medium.

Cell Viability Assessment by MTT Assay

For each experiment, HepG2 cells (10⁴ cells/well) were seeded on 96 well plates and allowed to adhere overnight. When cells reached 60-70 % confluent, the medium was then aspirated off. Non-attached cells were removed by rinsing the cultures in PBS. Cells were treated with increasing concentrations of xenobiotic (100 µl/well) and incubated for the required time (24 h). 10 µl of MTT reagent (5 mg/ml PBS) were added to the cultured medium in each well. After 3 h of incubation at 37 °C, culture media were cautiously removed and of DMSO (100 µl) were added to each well in order to dissolve the insoluble formazan crystal. Plates were read 540 nm. All experiments were conducted three biological and technical replicates.

Microscopic Examination of ROS

HepG2 cells (5x10⁴) were seeded in 6-well plate; the medium was replaced with the test compounds. HepG2 cells were treated with a control vehicle and xenobiotics. For a positive control, cells were treated with 400 µM hydrogen peroxide (H₂O₂) for 3 h. The cells were then rinsed with PBS and incubated with 25 µM DCF for 45 minutes at 37 °C. The cells were then washed with sterile PBS and observed under microscope. All images were acquired using identical settings.

Quantification of Intracellular ROS by ROS-Glo™ H₂O₂ Assay

H₂O₂ as marker of ROS was measured using ROS-Glo™ H₂O₂ assay kit from Promega following the manufacturer's instructions. In brief, HepG2 cells were plated in a white 96-well plate at density 1x10⁴ cells per well. Cells were exposed to the experimental compounds for the required time and as described by the instructions. Plate was returned to the incubator for the final 6 h of treatment. Luminescence was measured using plate reader.

RNA Extraction and RT-PCR

In accordance to the manufacturer's protocol RNeasy Plus Mini Kit (QIAGEN) was used and total RNA was extracted from HepG2 cells. To check the integrity of RNA, RNA integrity number (RIN) was measured using 28S to 18S rRNA ratio. All RNA was reverse-transcribed into cDNA using ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) primers (INTEGRATED DNA TECHNOLOGIES) were selected for metallothionein 2A (MT2A) and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH):

| Gene | Forward Sequence | Reverse Sequence |
|-------|------------------------------|------------------------------|
| MT2A | 5'-CCGACTCTAGCCGCTCTT-3' | 5'-GTGGAAGTCGCGTTCTTTACA-3' |
| GAPDH | 5'-GTCTCCTCTGACTTCAACAGCG-3' | 5'-ACCACCCTGTTGCTGTAGCCAA-3' |

PCR was carried out to determine the effect of cisplatin and/or zinc sulphate cytotoxicity treatments on gene expression of MT2A against the reference gene (GAPDH) in HepG2 cells. Real-time PCR (RT-PCR) was carried out using QuantiFast SYBR Green PCR Kit. RT-PCR using the MT2A was made in one cycle of 5mins at 95°C followed by 40 cycles of 10secs at 95°C and 30secs at 58°C. A final extension was carried out for 1 min at 58°C and 30secs at 95°C.

Statistical Analysis

The software package GraphPad (version 6.0, Prism, CA, US) was used to plot the dose-response curves, as well as to calculate the IC_{50} values. A minimum of three independent experiments were carried out and the standard error of the mean (SEM) derived. Statistical significance was determined using the most relevant statistical test, as indicated, where $P < 0.05$ was considered to be significant.

3. RESULTS

The influence of cisplatin on HepG2 cell line viability

The MTT assay results (Fig. 1) showed that cisplatin decreased HepG2 cell viability by exposing to increasing concentrations. After 24 h of cisplatin treatment, significant cell death was seen starting from 5 μ M. Cells were 100 % viable in the vehicle control DMSO (0.1 %). The calculated IC_{50} was 10 μ M.

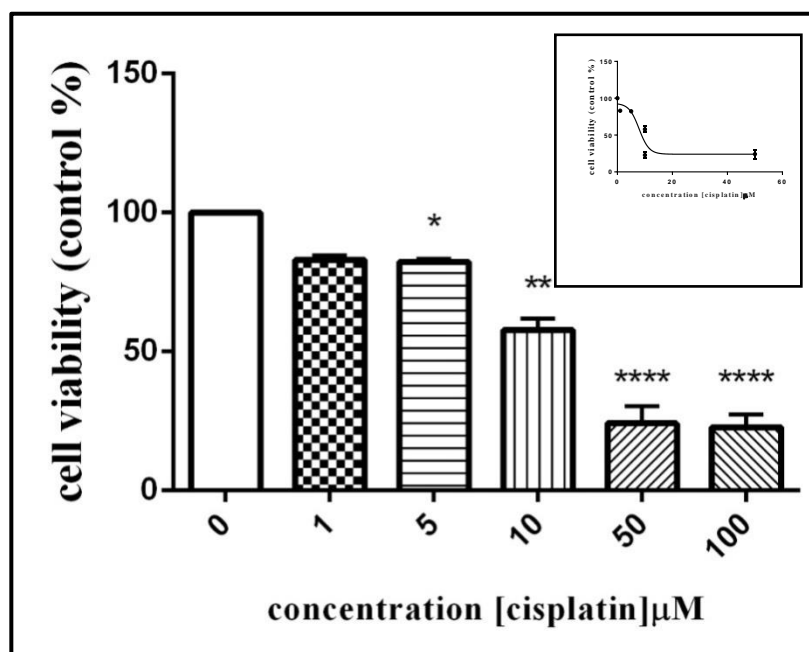


Figure 1 The effect of cisplatin on HepG2 cell viability. HepG2 cells were cultured in 96 well plate (1×10^4) and incubated overnight. HepG2 cells were treated with increasing concentrations of cisplatin (24 h). The MTT assay was performed to examine cell viability. Three independent experiments were carried out. The mean values were calculated and plotted as % viability of the control (defined as 100% viability) and error bars represent SEM. Data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and P values are shown where the difference between responses of different concentrations relative to the vehicle control were statistically significant. * $P < 0.05$ ** $P < 0.01$ and **** $P < 0.00001$.

The influence of zinc sulphate on HepG2 cell line viability

Cells were seeded and three different concentrations of zinc sulphate were chosen, 5 μ g/ml, 10 μ g/ml and 15 μ g/ml. HepG2 cells were incubated with zinc sulphate for 24 h. Incubation of HepG2 cells with zinc sulphate for 24 h did not affect cell viability (Fig. 2). Cell viability after treating cells with 5 μ g/ml and 10 μ g/ml were 97 % and 88 %, respectively. For subsequent experiments, two concentrations (5 μ g/ml and 10 μ g/ml) were chosen.

Effect of co-treatment of cisplatin and zinc sulphate

In order to examine the effect of zinc sulphate versus cisplatin cytotoxicity, cells were preincubated with zinc sulphate for 24 h. Cells were then incubated with cisplatin for another 24 h. The MTT results (Fig. 3) for the HepG2 cells pre-exposed to 5 μ g/ml zinc sulphate indicated significantly enhanced cell viability than HepG2 cells treated with cisplatin alone.

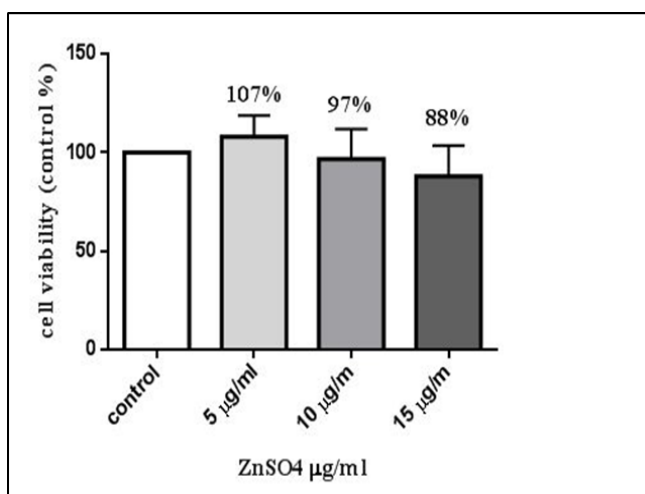


Figure 2 The effect of zinc sulphate on HepG2 cell line viability. HepG2 cells were seeded in 96 well plate (1×10^4) and incubated overnight. HepG2 cells were treated with three concentration of zinc sulphate and incubated for 24 h and the MTT assay was carried out to assess cell viability. Three independent experiments were carried out. The mean values were calculated and plotted as % viability of the control (defined as 100% viability) and error bars represent SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

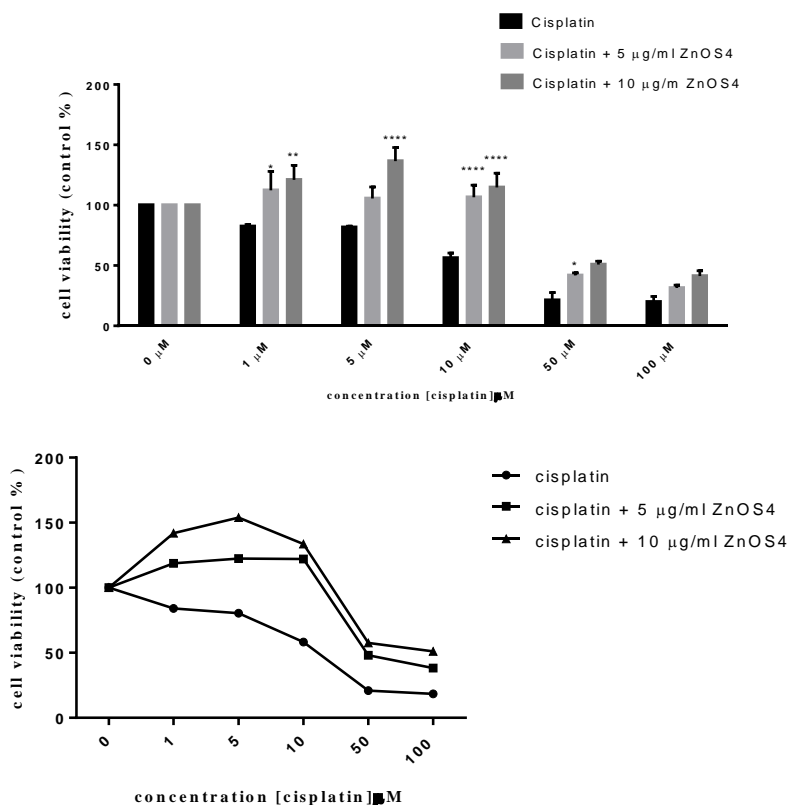


Figure 3 Co-exposure of HepG2 cells to Zn and cisplatin. HepG2 cells were seeded in 96 well plate (1×10^4) and incubated overnight. HepG2 cells were treated with 5 µg/ml and 10 µg/ml zinc for 24h. Cells were then treated with increasing concentrations of cisplatin for 24 h. The MTT assay was carried out to assess cell viability. Three independent experiments were carried out. The mean values were calculated and plotted as % viability of the control (defined as 100% viability) and error bars represent SEM. Data were statistically analyzed using two-way ANOVA followed by Tukey's multiple comparison test and P values are shown where the difference between responses of different treatments were considered to be statistically significant *P < 0.05, **P < 0.01, and ****P < 0.0001.

Quantification of ROS levels

To measure the level of ROS in HepG2 cells exposed to either cisplatin, zinc sulphate or both, the ROS-Glo™ H₂O₂ assay was used. The results (Fig. 4) show that HepG2 cells treated with cisplatin (1 μM and 5 μM) displayed a significant ($P < 0.01$) higher concentration of ROS when compared to control cells. Consistent with the MTT results, HepG2 cells preincubated with zinc sulphate and then treated with cisplatin showed a significant reduction in ROS levels.

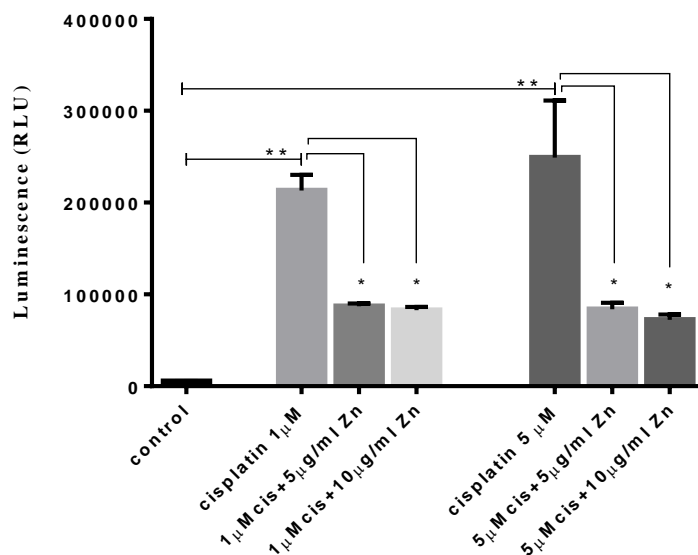


Figure 4 Measurement of ROS levels in HepG2 cells. HepG2 cells were seeded in 96 well plate (1×10^4) and incubated overnight. HepG2 cells were treated with 5 μg/ml and 10 μg/ml zinc for 24h. Cells were then treated with 1 μM or 5 μM cisplatin for 24 h. The mean values were calculated and plotted, and the error bars represent SEM. Data were analysed using one-way ANOVA followed by Tukey's multiple comparison test and P values are shown where the difference between responses of different treatments were determined to be statistically significant * $P < 0.05$ and ** $P < 0.01$.

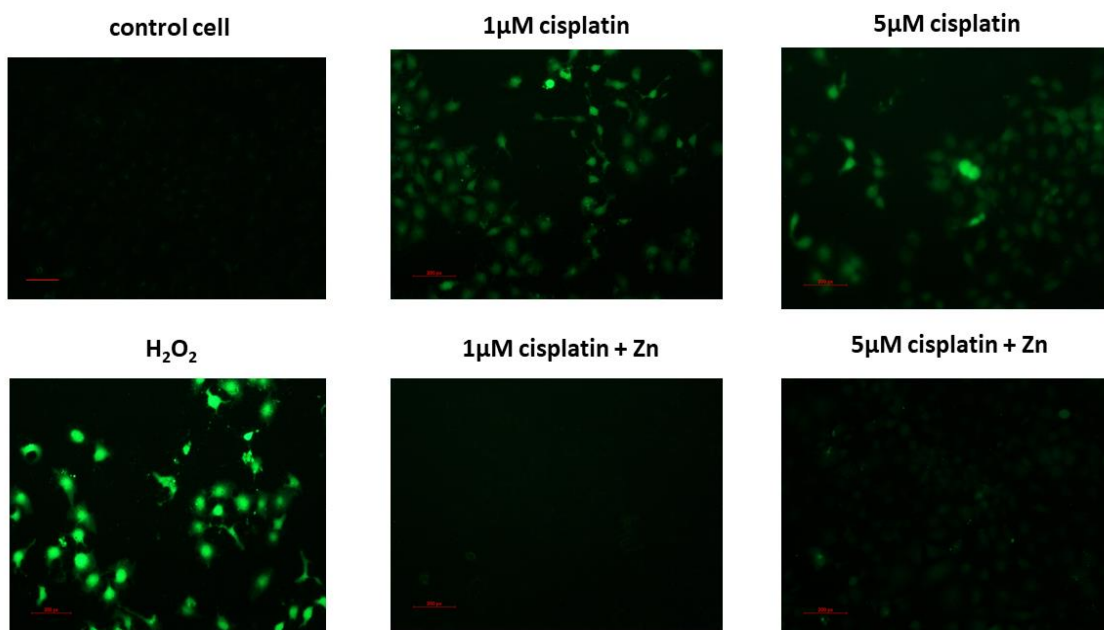


Figure 5 ROS detection by DCF staining. HepG2 cells were seeded in 6 well plate and incubated overnight. HepG2 cells were treated with 5 μg/ml zinc for 24h. Cells were then treated as described in materials and methods. Cells were fixed and stained with 25 μM DCFH-DA. Cells were imaged with fluorescent (green) light. Identical settings were used for all images. Higher fluorescence

intensities correspond with higher levels of ROS production. All images were taken using a fluorescence microscope. Scale bar = 200 μm .

Microscopic examination of ROS

Intracellular ROS generation (Fig. 5) was detected by the highly fluorescent 2', 7'-dichlorofluorescein (DCF) staining and visualized by fluorescence microscopy. The results show that there is an excess in intracellular ROS apparent by a greater in green fluorescent in cells treated with cisplatin. However, the green fluorescent reduced to a negligible level in cells pretreated with Zn. Cells treated for 3 h with 400 μM H_2O_2 was used as positive control.

Metallothionein expression

RNA extraction and RT-PCR were carried out in HepG2 cells to determine the effects of cisplatin and/or zinc sulphate treatments on gene expression of MT2A against a reference gene (GAPDH). HepG2 cells were treated with two concentrations of cisplatin (1 and 5 μM). In addition to cisplatin, cells were also treated with zinc sulphate (5 $\mu\text{g}/\text{ml}$) only and in combination with cisplatin. RIN for all samples was checked and the value ranges between (9.8-10).

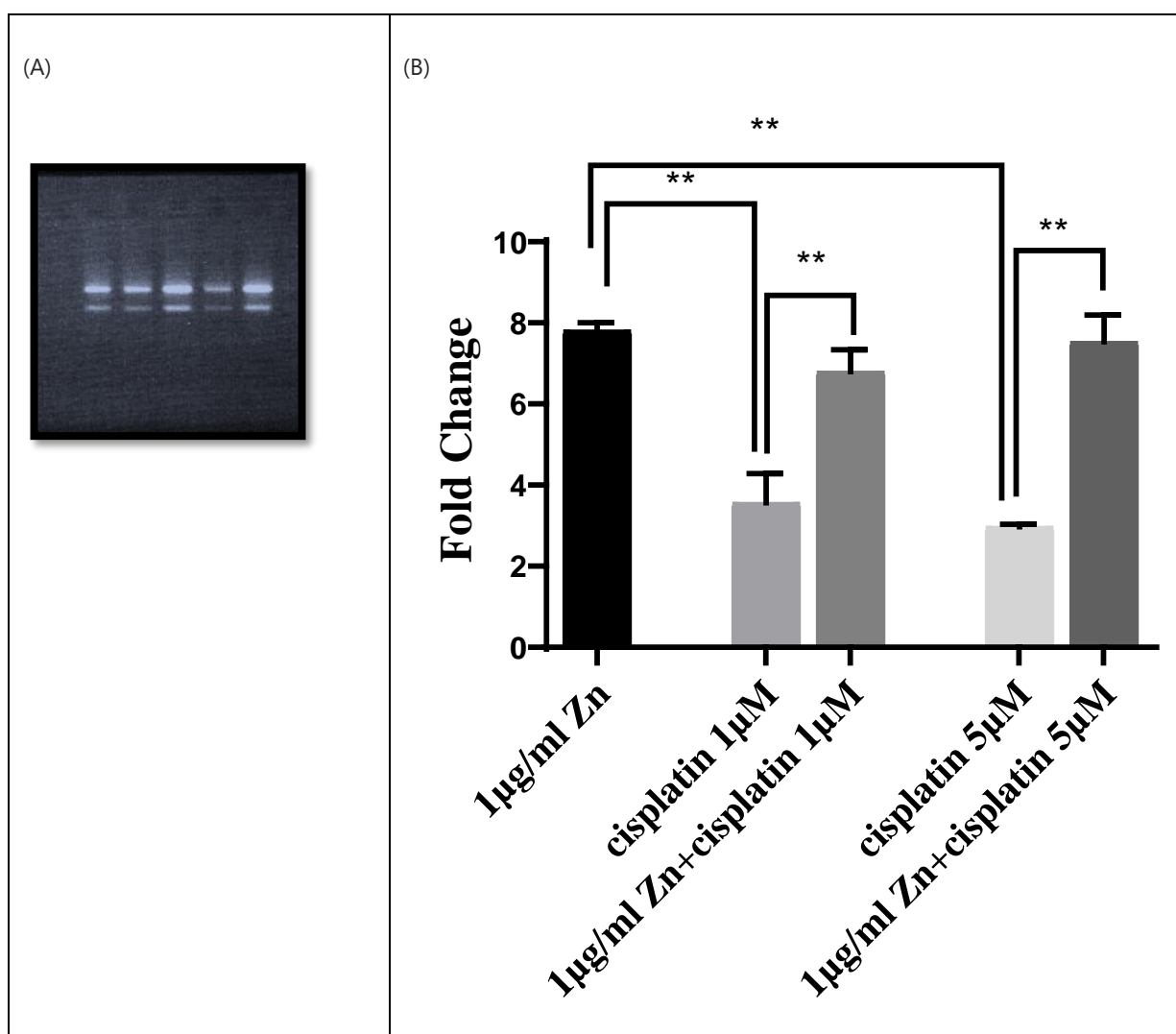


Figure 6 Expression pattern of MT2A gene during five different treatment conditions. HepG2 cells were seeded and incubated overnight. HepG2 cells were treated with 5 $\mu\text{g}/\text{ml}$ (Zn) for 24 h. Cells were then treated with two concentrations of cisplatin (1 and 5 μM) for 24 h. (A) represents the quality of extracted RNA on agarose gel. (B) Fold change graph represents the comparative expression quantification of gene (test vs control). Three independent experiments were carried out. RT-PCR data was analyzed by $\Delta\Delta\text{CT}$ method.

The data (Fig. 6) showed that MT2A gene expressions is increased significantly in cells treated with Zn when compared to cells treated with cisplatin only. However, exposing to cisplatin also resulted in upregulation of MT2A gene. In the combination treatment, MT2A gene expression was found to be upregulated in both treatments significantly when compared to cells treated with cisplatin alone. No significant differences seen between cells treated with zinc alone and cells pretreated with zinc and the cisplatin.

4. DISCUSSION

In the present study, cytotoxicity induced by cisplatin was assessed by MTT assay, which showed that treatment of the cells with increasing concentrations of cisplatin ranging between 0-100 μ M resulted in a significant reduction of cell viability. Many studies have reported the same result, that cisplatin induced toxicity in a concentration dependent manner (Serpeloni et al., 2012; Waseem et al., 2015), and in a time dependent manner (Bennukul et al., 2014). These results are similar to those seen in rat primary hepatocytes where cisplatin induced cell death in a dose-dependent manner (Cho et al., 2012). A previous study performed in ICR mice treated with a single high-dose of cisplatin (45 mg/kg body weight) over a 16 h resulted in prominent liver injury, characterized by histopathological and biochemical changes including elevation in the serum ALT and AST levels, and in damage in liver antioxidant machinery (Gong et al., 2015). Another *in vitro* study has also reported similar damages induced by cisplatin (Park et al., 2009).

Zinc is an essential mineral that is necessary for functioning of various proteins as well as maintenance of the human health. Our results regarding the effect of zinc on HepG2 cell line viability showed that incubation of HepG2 cells with the different concentrations of zinc sulphate 5 μ g/ml, 10 μ g/ml and 15 μ g/ml for 24h did not affect cell viability. Previous studies have shown similar results, that low zinc sulphate concentrations (5-10 μ M) had no effect on cell viability (Ibbitson, 2012). Conversely, HepG2 cells exposed to relatively high concentrations of zinc sulphate (from 25 to 125 μ M) for 24 h showed a decrease in the cell viability in a dose dependent manner (Zheng et al., 2013). This could indicate that zinc has a protective effect at low concentration, whilst high concentrations of zinc can negatively affect cell viability. The recommended dose of zinc ranges between 11-19 mg/day, therefore, the concentrations used in this study are clinically relevant.

Previous studies have demonstrated that cisplatin can cause nephrotoxicity and hepatotoxicity due to oxidative stress (Pratibha et al., 2006; Santos et al., 2007; Chirino et al., 2009). Available data from previous studies has proved that pre-treatment with antioxidants can scavenge free radicals and ameliorate cisplatin induced toxicity (Mansour et al., 2006; Kuhad et al., 2007). The present study demonstrated that HepG2 cells pre-treated with zinc sulphate showed enhanced cell viability compared to HepG2 cells treated with cisplatin alone. Many studies confirm that zinc sulphate can work as MT inducer, which can act as free radical scavenger and can reduce oxidative stress caused by cisplatin (Satoh et al., 1997; Kalsotra et al., 2018) and this is consistent with the results presented here. This observation of reduced cytotoxicity due to pre-treatment with Zn may bring to light the importance of giving small doses of trace metals (like zinc) to cancer patients who are treated with cisplatin to reduce the cisplatin-induced toxicity.

The production of ROS and the resultant oxidative stress has been shown to generally be the cause of many drug toxicities. Quantification of ROS levels was carried and the level of H₂O₂ was determined. Measuring H₂O₂ is one of the most accurate and easiest ways to quantify ROS levels (Kelts et al., 2015). In this study we used the ROS-Glo™ H₂O₂ assay, which has been used in many studies and has produced precise results (AlGhamdi et al., 2015; Kato et al., 2017). The results (Fig. 4) showed that HepG2 cells treated with cisplatin (1 μ M and 5 μ M) displayed a significantly higher concentration of ROS when compared to control cells. Consistent with the MTT results, the HepG2 cells preincubated with zinc and then treated with cisplatin showed a significant reduction in ROS production. This indicated that co-treatment of zinc could effectively conquer the generation of ROS which fits with several previous studies (Du et al., 1994; Chimienti et al., 2003). It has been shown that increased levels of intracellular zinc can stimulate MT induction (Ohly et al., 2002; Zheng et al., 2013). These observations could indicate that zinc protects against cisplatin cytotoxicity through inhibiting oxidative damage.

To uncover the possible molecular basis of the cytotoxicity, MT specifically the MT2A gene, was examined by RT-PCR. MT2A was expressed in cells that were treated with cisplatin and zinc sulphate individually or in a combination. The MT gene expression results showed higher expression level of MT2A in HepG2 cells that were treated with zinc sulphate compared to cells that were treated with cisplatin, which confirm that zinc sulphate works as a MT inducer (Pérez & Cederbaum, 2003). Having shown MT2A expression in cells treated with cisplatin may indicate the role of ROS in sensitizing cells to induce expression of MT genes. Our data also showed that expression of the MT2A gene was significantly higher (in terms of fold change) in cells pretreated with zinc, suggesting a protective role of MT2A which agrees with the results of other studies (Chung et al., 2006; Al Ghamdi et al., 2015). These findings agree with the previous studies that have shown over expression of MT2A protects Huh7 cells against cytotoxicity induced by doxorubicin (Al Ghamdi et al., 2015). Upregulation of MT2A was reported to be induced in response to cisplatin exposure in rat

primary hepatocytes (Cho et al., 2012). Other drugs can induce MT expression include alcohol and acetaminophen (Liu et al., 1999; Wang et al., 2005), and in rat hepatocytes MT had a defending mechanism against carbon tetrachloride-induced hepatotoxicity (Di Silvestro and Carlson, 1992), suggesting a strong relationship between increased MT synthesis and decreasing of oxidative stress. In clinical studies, enhanced expression of MT predicts chemo-resistance in breast cancer patients (Saika et al., 1994). Naganuma and his group found that pre-induction of MT synthesis reduced a lethal toxicity, bone marrow toxicity and nephrotoxicity of cisplatin in mice (Sato et al., 1988). These results suggest that overall zinc acts as a protective factor against cisplatin-induced hepatotoxicity through reduction of ROS production by inducing MT2A expression. Supplements of cancer patients with antioxidants during treatment course are still controversial. At the Mayo Clinic (USA), a cohort study has been conducted on 1129 patients diagnosed with lung cancer, revealed that the rate of mortality was decreased by 26% in patients taking a micronutrient supplement compared with patients not taking supplements (Jatoi et al., 2003). Reducing cisplatin cytotoxicity is a novel approach. Further studies are needed on the efficacy and safety of supplementing patients with antioxidants.

5. CONCLUSION

Oxidative stress plays an important role in cisplatin-induced toxicity. Treating cells with zinc sulphate generally showed a protective effect against cisplatin-induced hepatotoxicity. The protective effect of zinc sulphate is associated with its antioxidant properties, this might occur through induction of MT2A. Our study has clearly demonstrated that MT induction could protect against cisplatin-induced hepatotoxicity.

Declaration of interest

The authors report no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome. The authors alone are responsible for the content and writing of this article. As a corresponding Author, I confirm that the manuscript has been read and approved for submission by all the named authors.

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List of Abbreviations

| | |
|---------------|--|
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| DCF | Dichlorofluorescein diacetate |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase. |
| H2O2 | Hydrogen peroxide |
| MT | Metallothionein |
| MT2A | metallothionein 2A |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| PBS | Phosphate buffered saline |
| RIN | RNA integrity number |
| ROS | Reactive oxygen species |
| RT-PCR | Real time polymerase chain reaction |
| SEM | Standard error of mean |
| Zn | Zinc |

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