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ORIGINAL RESEARCH article

Metronidazole-induced neurotoxicity: Is iron a contributing factor?

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Abstract: Metronidazole-induced neurotoxicity is a rising challenge in managing susceptible infections. The mechanisms involved in metronidazole-induced neurotoxicity are not fully unraveled. This study aimed to explore the effect of metronidazole on iron homeostasis in SH-SY5Y neuroblastoma cells. Confluent SH-SY5Y neuroblastoma cells were treated with different concentrations of 1.0, 10, 25, 50, 100, and 250 µM of metronidazole only or in combination with 20 µM iron. DMSO or culture media was used as control. Viability and ferritin assays were conducted on the treated cells. The treatments were for 24 hours, 48 hours, and 72 hours, respectively. In the viability assay, doses of metronidazole reduced the viability of SH-SY5Y neuroblastoma cells in a time and concentration-dependent manner. After 24-hour treatment, 250 µM metronidazole significantly reduced cell viability while 50 µM, 100 µM and 250 µM metronidazole reduced considerably viability only after 48-hour and 72-hour compared with control. Different doses of metronidazole 50 µM, 100 µM, and 250 µM in 20 µM iron reduced viability in a time-dependent manner in all the test periods. Metronidazole also induced a time- and concentration-dependent increase ($p<0.05$) in cellular iron uptake in the 48-hour and 72-hour treated cells in concentrations above 25 µM metronidazole. It is concluded that metronidazole induces a time and concentration-dependent iron overload and consequent cell death in SH-SY5Y neuroblastoma cells and this may contribute to the mechanism of metronidazole-induced neurotoxicity.

Introduction

In vitro cell culture techniques have successfully been developed in the neurobiology field to address specific questions of cell biology and nervous system functioning. This has formed a basis to systematically study the nervous systems, revealing the complexity of cellular functions in the central nervous system (CNS). It has also provided a convenient experimental tool for studying defects and disease processes in the CNS [1]. Various techniques abound for the study of neurotoxicity using dedicated cell lines. One of such dedicated cell lines is the human neuroblastoma SH-SY5Y cells. SH-SY5Y neuroblastoma cells are a replicated offspring of SK-N-SH cells originally isolated from a bone marrow biopsy of a neuroblastoma patient in the early 1970s [2]. These cells consist of adherent and floating cells bearing two morphologically distinct phenotypes (neuroblast-like cells and epithelial-like cells) which can be differentiated into a more mature neuron-like phenotype characterized by neuronal markers [3]. Moreover, they possess many biochemical and

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functional properties of human neurons, including several transport proteins for neurotransmitters and other biomolecules like the divalent metal transporter 1 (DMT1) and ferropotin (IREG1) [4].

Iron is an essential molecule with pro-oxidant characteristics, it catalyses the synthesis of hydroxyl radicals in the intracellular environment owing to its reductive role in the Fenton reaction [5, 6]. The brain consumes the largest amount of total oxygen in the body due to its high respiratory or oxidative metabolic ability, consequently, it generates a large volume of reactive oxygen species [7]. Brain iron is mainly bound to ferritin and only unbound iron is physiologically active in the brain. Hence ferritin is recognised as the protein responsible for brain iron homeostasis. Search for the mechanism of metronidazole-induced neurotoxicity is still on as the various proposed mechanisms have not answered all the questions arising from its occurrence. One of the proposed mechanisms of metronidazole-induced neurotoxicity is interference with oxidative processes in the nervous system [8-11]. Metronidazole is a prodrug that undergoes reductive activation mediated by pyruvate: ferrodoxin oxidoreductase (PFOR) whose expression is positively regulated by iron [12]. Iron is ubiquitous and occurs in high abundance in the CNS. Iron overload is implicated as a major contributor to neurotoxicity and neurodegeneration as evidenced in several neurologic conditions [13, 14]. This study aimed at exploring the effect of metronidazole on iron uptake in the human neuroblastoma SH-SY5Y cells.

Materials and methods

Cell line: Human neuroblastoma SH-SY5Y cells (CRL-2266, ATCC' Rockville, USA) were used for the study and they were purchased from Gibco, USA. Their use required no institutional ethical approval.

Cell culture: Unless stated otherwise, cells were grown in Dulbecco's minimum essential medium in Han's F12 media at pH 7.4, supplemented with 10.0% fetal bovine serum, 01.0% penicillin/streptomycin solution and maintained at 37°C in 5.0% CO_2 at constant humidity, refreshing the medium every four days until the cells were confluent. Differentiation of the cells was initiated 48 hours after the initial plating by substituting the growth medium with serum-free neurobasal medium (containing B27 supplement and GlutaMAX) and 10 µM all-trans-retinoic acid (ATRA). The cells were allowed to grow in an ATRA-containing neurobasal medium for five days, refreshing the medium every 48 hours [15]. The confluent cells were subsequently passaged to maintain their viability and increase the quantity available for experiments.

Treatment of cells with metronidazole: Completely differentiated cells grown in 96-well plates were removed from the incubator, transferred to the hood, and the culture media removed. A quantity (200 µL) of each designated concentration (1.0, 10, 25, 50, 100, and 250 µM) (based on clinical relevance) of the test media was added to each of the six replicate wells using a multichannel pipette. The plates were incubated for 24-hour, 48-hour and 72-hour in the cell culture incubator. DMSO (200 µL) was used as a negative control in the experiments. Treatment media was refreshed for the plates in the 48-hour and 72-hour groups. After the desired incubation period, the plates were removed from the incubator and the test media was aspirated and the cells were harvested for endpoint assays to determine the effects of treatment on the cells. Protein quantification was done using the bicinchoninic acid assay method.

Viability assay of SH-SY5Y following treatment with metronidazole: The reagents for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay were prepared according to the manufacturer's instructions. After incubating the plates for 24, 48, or 72 hours, respectively in the test drug, they were removed from the cell culture incubator and washed with DMSO. Each well was treated with MTT 20 µL/well and incubated for four hours at 37°C. After the incubation period, the plates were removed from the incubator, the media was gently aspirated into the bleach bottle without disrupting the cell monolayer. An aliquot (100 µL) of DMSO was added to each well and mixed properly with a pipette. The wells were covered with foil to

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avoid light and incubated in a rotary shaker for 15 min. The plates were then removed from the shaker and the absorbance was measured at 570 nm in a spectrophotometer (Thermo-Fischer, UK) [16].

Effect of metronidazole on iron uptake: A standard iron solution (1.0 M) was prepared by dissolving 1.52 g ferrous sulphate powder in 10.0 mL 0.1 M HCl. Completely differentiated cells were incubated in serum-free minimum essential medium (MEM) at pH 5.8 for 24 hours at 37.0^{o}C. After the incubation period, the cells were treated with filtered aliquots of metronidazole in MEM plus prepared iron solution to give a final concentration of the different test conditions in 20.0 µM elemental iron and all the wells buffered to pH 5.8. A positive control (iron solution in MEM) and negative control (MEM only) were also included in the plates. The plates were incubated on a rotary shaker (6.0 *g*) at 37^{o}C for 24, 48, and 72 hours and the media refreshed for the 48-hour and 72-hour groups respectively. On completion of the incubation period, the plates were removed, and the media aspirated into the bleach bottle. The plates were washed twice with wash solution and the media was replaced with serum-free MEM and incubated for 24 hours. After incubation, the cells were washed, and lysed and the supernatant was collected in aliquots for ferritin ELISA and bicinchoninic acid (BCA) assay [16]. The plates for MTT were not lysed after washing rather, they were treated with MTT reagent and assayed as previously described.

Ferritin assay: The manufacturer's instructions were followed in the preparation of reagents and standards for the ferritin assay. Ferritin standards (50.0, 25.0, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.00 ng/mL) were prepared by serial dilution method starting from 50 ng/mL stock. An aliquot (50 µL) of ferritin standard or sample was added in duplicates per well and the plate was tapped gently to thoroughly coat the wells and incubated for two hours at room temperature. The plate was washed five times with 200 µL of the wash buffer and gently tapped on an absorbent paper towel to completely remove the liquid after the last wash. The biotinylated ferritin antibody (50.0 µL) was added to each well and incubated for one hour at room temperature. The plates were washed five times as previously done followed by the addition of 50.0 µL SP-conjugate to each well. All bubbles were removed by tapping and the plates incubated at room temperature for 30 min. After the incubation, each plate was washed as above. Some chromogen substrate (50.0 µL) was added to each well and spread well by tapping to remove bubbles, then each plate was incubated at room temperature for 12 min. At the end of the incubation, 50.0 µL of the stop solution was added to each well and the plates read immediately at 450 nm (MicroPlate Reader 550, Bio-Rad Laboratories, Denmark) [17, 18].

Statistical analysis: Data are presented as mean±standard error of the mean. Statistical analysis was done using one-way ANOVA followed by Dunnet's post hoc test for multiple comparisons (GraphPad Prism 6 Software, San Diego California USA). Correlations for protein analysis were calculated by Pearson's test. A statistical difference between the compared data was considered significant at $p<0.05$.

Results

Metronidazole reduces the viability of SH-SY5Y cells dependent on time and concentration: In the cell viability experiments, metronidazole exhibited time and concentration-dependent effects on the treated cells, the viability of the treated cells reduced with an increase in metronidazole concentration, the 250 µM treated cells were the least viable (<10.0% viability across all treatment periods). The effect of treatment duration on cell viability of identical metronidazole concentration was marginal at concentrations of 25.0 µM and below. After 24-hour treatment, 250 µM metronidazole reduced significantly cell viability compared with the growth media-treated cells (**Figure 1**). Cell viability ranged between 8.9% to 83.2% after 48-hour treatment (**Figure 2**) with a significant reduction in viability for the 50 µM, 100 µM, and 250 µM treated cells. These effects were similar in the 72 hours treated cells with viability ranging between 07.5% to 78.5% (**Figure 3**).

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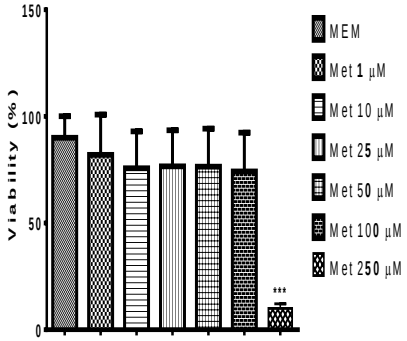
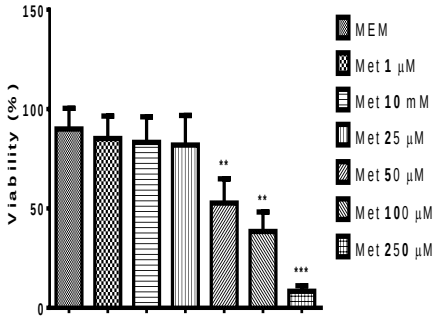
Figure 1: Cell viability after 24 hrs treatment with metronidazole



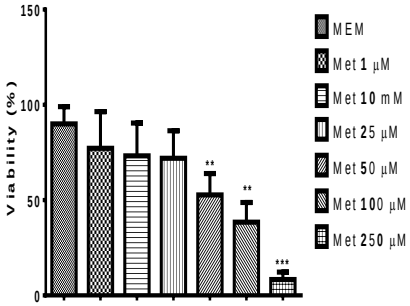
$^{***}p<0.001$ with minimum essential media (MEM), Met: metronidazole

Figure 2: Viability data of SH-SY5Y cells treated with metronidazole for 48 hrs



$^{**}p<0.01$ *and* $^{***}p<0.001$ with minimum essential media (MEM), Met: metronidazole

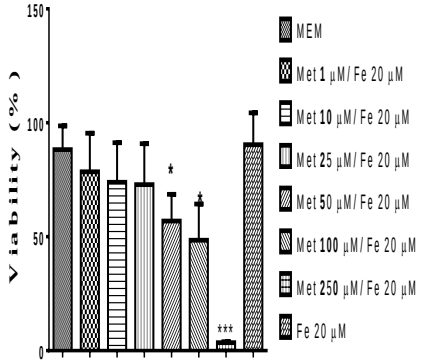
Figure 3: Viability data of SH-SY5Y cells treated with metronidazole for 72 hrs



$^{**}P<0.01$ *and* $^{***}P<0.001$ with minimum essential media (MEM), Met: metronidazole

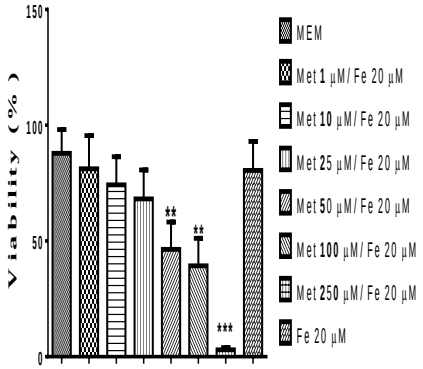
Metronidazole increases SH-SY5Y cell iron uptake: In determining the effect of metronidazole on cellular iron uptake, cytotoxicity experiments were conducted to determine the viability of SH-5YSY cells when concomitantly treated with the various concentrations of metronidazole and 20.0 µM ferrous sulphate. Treatment with metronidazole and ferrous sulphate yielded a concentration and time-dependent effect on the treated cells with cell viability reducing with an increase in dose and time of treatment. After 24-hour incubation, cell viability reduced significantly in the 50, 100, and 250 µM metronidazole treated wells (**Figure 4**). The effect of co-exposure of the cells to metronidazole and ferrous sulphate was more pronounced after 48 hours as shown in **Figure 5**. Metronidazole concentrations of 50.0 µM and above caused a significant reduction in cell viability. The reduction in cell viability seen after 72-hour incubation with metronidazole and ferrous sulphate was more intense with 100 and 250 µM concentrations (**Figure 6**).

Figure 4: Cell viability after 24 hrs incubation in ferrous sulphate and metronidazole



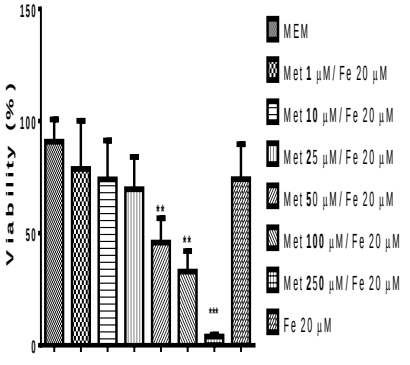
$^{*}P<0.05$ and $^{***}P<0.001$ compared with minimum essential media (MEM) only, Met: metronidazole, Fe: ferrous sulphate

Figure 5: Effect of metronidazole and ferrous sulphate after 48 hrs co-treatment on the viability of SH-SY5Y cells



$^{**}P<0.01$ *and* $^{***}P<0.001$ compared with minimum essential media (MEM). 4 x 10^3 cells/well. Met: metronidazole, Fe: ferrous sulphate

Figure 6: Effect of metronidazole and ferrous sulphate 72 hrs co-treatment on the viability of SH-SY5Y cells



$^{**}p<0.01$ *and* $^{***}p<0.001$ with minimum essential media (MEM), Met: metronidazole, Fe: ferrous sulphate

The total iron absorption data is shown in **Figures** 7, 8, and 9. Metronidazole induced a slight increase in iron uptake into the cells after 24-hour treatment although the changes were not statistically different from the iron-only treated cells (**Figure 7**). However, metronidazole induced a time and concentration-dependent increase significantly in cellular iron uptake in the 48 hour (**Figure 8**) and 72 hour treated cells in concentrations above 25.0 µM metronidazole. In the 72-hour treated group (**Figure 9**), 01.0 µM metronidazole-treated cells had reduced significantly iron absorption compared with the iron-only treated cells.

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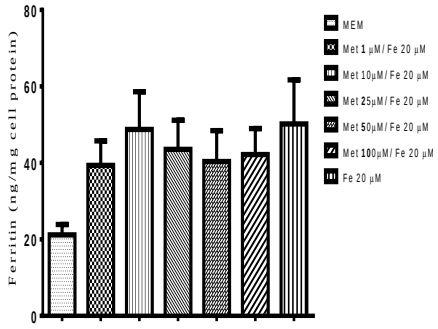
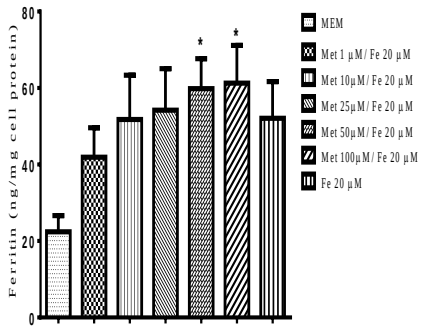
Figure 7: Iron absorption of SH-SY5Y cells following metronidazole, and ferrous sulphate for 24 hrs



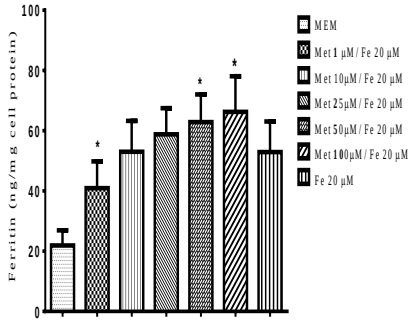
p>0.05 with the 20 µM iron-treated cells. Met: metronidazole and Fe: ferrous sulphate

Figure 8: Cellular iron absorption data following 48 hrs treatment with ferrous sulphate and metronidazole



*p<0.05 with the ferrous sulphate 20 µM only. Met: metronidazole and Fe: ferrous sulphate

Figure 9: Cellular iron absorption data after 72 hrs with ferrous sulphate and metronidazole



*p<0.05 with the ferrous sulphate 20 µM only. Met: metronidazole and Fe: ferrous sulphate

Discussion

Neurotoxicity arising from the use of metronidazole is a concern in clinical practice. Unraveling the mechanism of metronidazole-induced neurotoxicity is necessary to prevent injury and maximize therapy. Several attempts have been made to demystify the mechanisms responsible for its neurotoxic effect. The induction of nitric oxide synthase, B-cell lymphoma-2- associated-X protein (Bax), and caspase three protein expressions and reduction of B-cell lymphoma-2 (Bcl-2) and endothelial nitric oxide synthase expression in brain tissue with an increase in serum inflammatory markers in rats [19]. Many of the available reports postulate that interference with oxidative processes is a major mechanism for the neurotoxicity induced by metronidazole [9-11]. However, there is a paucity of studies directed at metronidazole-induced neurotoxicity using cell cultures. Although the various effects, targets, and modes of action of metronidazole are well characterized in bacteria, the consequences and how metronidazole interacts with mammalian cells are still not fully deciphered. In this study, human neuroblastoma (SH-SY5Y) cells were employed to elucidate the effect of metronidazole on cell viability and iron uptake. This cell line has been extensively used as a neuronal model since the early 1980s, as it possesses many biochemical and functional properties of human neurons [4, 20]. They are useful in assessing morphological and many differentiated neuronal functions hence their usefulness in neurotoxicity screening of parent drug molecules [21]. Although the SH-SY5Y cell line has found invaluable use in neurotoxicity studies, it has also some limitations that need to be considered while making a choice. The SH-SY5Y cells are offsprings of tumour cells and may have the propensity to deviate from the expected outcomes. Whereas the nervous system is highly complex with multiple interactions between neurons and glia and several systems concurrently, the microenvironment of the cultured cells is unable to mimic these interactions [22]. These cells also exhibit unsynchronized cell cycles, possess several phenotypes, and lack excitability in the undifferentiated state [15]. However, this problem has been taken care of by the use of differentiation agents like retinoic acid.

Cytotoxicity testing is an essential procedure in cell-based neurotoxicity testing. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay is a high throughput screening model for the determination of cell viability in toxicity testing [22]. Healthy growing cells with intact metabolic capacity convert MTT to an insoluble, purple-colored formazan product that accumulates inside cells. Dead cells lose the capability to convert MTT to formazan, hence formation of the insoluble color serves as an accessible and convenient marker for healthy growing cells [24]. The quantum of formazan formed is directly proportional to the number of healthy growing cells as determined by the optical density at 570 nm using a plate reading spectrophotometer [25]. In this study, 50.0 µM metronidazole and concentrations above it exhibited a time and concentration-dependent effect on the treated cells. There is a paucity of reports on the outcome of

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metronidazole treatment on SH-SY5Y cells. Metronidazole-induced apoptosis of neuronal cell cultures by increasing the expression of annexin-v at a concentration of 40.0 µg/m [26].

Iron is vitally important for most life forms and is broadly utilized by different proteins to execute several functions including respiration, metabolism, and synthesis of new proteins, in essence, iron is essential for the survival of living systems [27]. Dysfunction in iron metabolism has been implicated in several disease states [28, 5]. Normally, iron is absorbed into cells using the DMT1, which is a steadfastly modulated process. Additionally, a new IREG1 has been identified recently. It is reported that both DMT1 and IREG1 exist in the CNS is expressed in neurons, glial cells, and astrocytes, they are thought to be responsible for maintaining iron homeostasis in the CNS [29, 30]. These transporters have been characterized also in SH-SY5Y cells, and IREG1 is thought to mediate iron efflux while DMT1 is thought to mediate iron influx in SH-SY5Y cells [31]. Excessive iron aggregation can activate oxidative stress and cell damage as it reacts with hydrogen peroxide to produce the hydroxyl radical and other ROS in the Fenton reaction [5, 6]. Iron accumulation and reduced viability of SH-SY5Y cells have been reported recently following treatment with iron (1.5-80 µM). Also, DMT1 expression decreased but persisted even at 80 µM while IREG1 expression increased with increased cellular iron content [31]. This implies that iron influx did not stop completely even at very high cellular iron concentration though an increased efflux process seemed to have been activated. In this study, the presence of metronidazole caused a drastic decline in cell viability compared to the cells treated with only iron. It is possible to then suggest that metronidazole favors an increase in DMT1 expression as opposed to IREG1 expression in the presence of iron and subsequent oxidative stress and cell death arising from iron accumulation. Iron is stored in the cytosol or mitochondria as ferritin. Ferritin has recently been shown to play a role in iron homeostasis, immunomodulation, inflammation, and antioxidation [6]. Ferritin structurally consists of an outer shell known as apoferritin and a hollow inner core that serves as a store for iron. The apoferritin shell also contains ferroxidase which converts iron from its active ferrous (Fe^{2+}) form into the inactive ferric (Fe^{3+}) form for storage [32]. Experimental evidence implicates ferritin as the protein responsible for iron delivery to the brain [33]. In low iron conditions, the ferritin molecule is degraded by activation of the lysosomal-autophagy pathway where nuclear receptor coactivator-4 binds to ferritin and causes the release of iron into the cytosol [32, 33]. Elevated ferritin levels have been implicated in various neuropsychiatric and neurodegenerative diseases in recent times [13, 14]. In such cases, the storage capacity of ferritin is thought to be overwhelmed resulting in an increased concentration of unsequestered iron in the cytosol with resultant production of free radicals and oxidative stress [13, 33, 32]. Four-fold increase in ferritin levels in SH-SY5Y cells treated with iron (1.5-5.0 µM) and a ten-fold increase for iron concentrations higher than that resulting in a commensurate elevation of the labile iron pool [31]. Currently, not more than a three-fold increase in ferritin levels was observed even though the cells were treated with 20.0 µM iron. Moreover, in the cells treated with 1.0 µM metronidazole/Fe 20.0 µM, a decrease in ferritin levels was observed. This suggests that the inherent efflux mechanism manned by IREG1 was not affected by the low concentration of metronidazole and was able to regulate iron absorption. Metronidazole undergoes reductive activation to form reactive intermediates which are toxic to susceptible organisms in anaerobic conditions. Several enzymes suggested to be involved in the reductive activation of MTZ include the pyruvate: PFOR which catalyzes electron transfer via its iron-sulphur clusters resulting in the generation of nitro-radical anion as metronidazole serves as a receptacle of electrons released by the action of PFOR [12, 34]. PFOR plays a critical role in the conversion of pyruvate to acetyl-CoA for energy production in anaerobes. PFOR expression is thought to increase in the presence of iron, this is corroborated by the increased conversion of metronidazole to its active nitro-radical forms with a consequent increase in its antibacterial activity [12] and toxic radical production [35]. There was a concentration and time-dependent elevation of ferritin levels in the metronidazole-plus-iron treated cells compared to the iron-only treated cells. It is suggested that metronidazole induced iron overload as evidenced by the increased ferritin in the treated cells with a consequent decrease in cell viability.

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Conclusion: Metronidazole induced a time and concentration-dependent decrease in SH-SY5Ycell viability and an increase in ferritin concentration in the treated cells. Metronidazole induces iron overload and consequent cell death in SH-SY5Y cells and this may contribute to metronidazole-induced neurotoxicity.

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