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Toxic Potentials of Methamphetamine in Neuroblastoma Cell Line and its Effects on the Mitochondrial Activity

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The cytotoxicity potentials of methamphetamine (METH) is presumably associated with oxidative stress induced apoptosis, this study therefore, investigated the toxic potentials of METH in neuroblastoma cells and further determined it effects on the mitochondrial activity.
Human neuroblastoma SK-N-BE (2) cells cultured in DMEM/F12 were used in this study. The cells were treated acutely with methamphetamine (1, 5, 10, 20, and 50 µg/mL) over 24, and were allowed to recover from METH treatment over 48, 72, and 96 h. Cell viability study was done with Trypanblue exclusion assay. The cell proliferative characteristics of the neuroblastoma cell line were investigated by constructinga cell proliferation curve. Mitochondrial activity was assessed using the XTT Assay. Statistical analysis were done with Graph Pad prism and significant difference were considered at $p<0.001$, 0.01 and 0.05.
The result showed normal growth in the untreated neuroblastoma cell over the 96 h of monitoring. Following treatment with METH, significant decrease in cell growth was observed when treated acutely with 5 and 10 µg/mL METH and allowed till 72 and 96 h recovery period. The SK-N-BE (2) treated with increasing concentration of METH showed no significant difference in cell viability over

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the recovery period from METH exposure. Toxicity of SK-N-BE (2) cells was only observed when treated with 10 µg/mL of METH. Significant decrease in mitochondria activity was observed when the cells were treated with 5, 10, 20, and 50 µg/mL METH and allowed till 72 h recovery.
The result showed that METH is cytotoxic to the SK-N-BE (2) cells and the mechanism of toxicity might be associated with inhibition of mitochondrial activity.

Keywords: Cytotoxicity; METH exposure; neuroblastoma; neuroendocrine tumour.

1. INTRODUCTION

Neuroblastomais a neuroendocrine tumour that arises from the sympathetic nervous system [1], with most of the lesions from neuroblastoma occurring in the medulla of the adrenalgland [2]. Neuro blastoma usually affects infants and young children, the occurrence of which is slightly higher among males than females. The etiology of neuro blastomastill remains obscure, environmental influences as well as parental exposures that highly impact disease occurrences have not been established [3]. Neuroblastomais a clinical problem, because the tumour is associated with clinical behavior ranging fromlife-threatening progression, maturation, and even spontaneous regression [2]. Their braod spectrum of clinical behaviours as aforementioned also includes differentiation to benignganglio neuroblastoma organglio neuroma, or to aggressive invasion with metastases to the liver, bone, bone marrow, and central nervous system (CNS) [4]. Neuroblastomais commonly presents between 18 and 22 months, with the majority of cases diagnosed prior to 5 years of age [5]. Age at diagnosis is an important indicator of the clinical course, infants less than 18 months of age are more likelyto spontaneous regression or become successfully treated with surgery alone whereas, older children are more likely to have aggressive tumors that are resistant to multimodal and cytotoxic therapies [5]. The survival of patients with high-risk neuroblastoma has improved throughout the years but most patients eventually die from relapse [4]. Relapsed neuroblastoma metastasizes to the CNS as one cause of death, with the overall incidence ofbrain metastasis in neuroblastoma after treatment ranging from 1.7% to 11.7% [4]. It is therefore imperative to develop more effective therapeutic strategies to further improve long-term survival ofpatients.

Methamphetamine (METH), is an extremely addictive pharmacologic psychostimulant with strong neurotoxic effects on the central nervous system (CNS) [6]. Methamphetamine is a member of the phenylethylamine class of psychostimulants, it has an added N methyl group which confers added lipid solubility, allowing for more rapid crossing of the BBB [7], (Vearrier et al., 2012).

This chemical compound rapidly enters and persists within the CNS for approximately 10 to 12 hours [8]. It is shown to result in excessive dopaminergic stimulationin the brain, as well as producing an imbalance in the release and re-uptake of dopamine, norepinephrine, and epinephrine [9]. Dopamine plays an important role in METH-induced toxicity, where it blocks the effect of certain antioxidants such as glutathione, leading to a build-up of reactive oxygen species, which can then result in neuronal apoptosis [10]. METH has the ability to alter the expression of several tight junction proteins and increases the permeability of brain-derived primary microvascular endothelial cells [11]. The mechanisms responsible for the damage caused by METH administration are complex and may involve various processes, thus oxidative stress is believed to be a prominent factor since METH induces reactive oxygen species such as hydroxyl radicals and superoxides that lead to cellular toxicity [12].

Apoptosis being defined as a distinct form of cell death, which can occur normally during development or occur as a defense mechanism when cells get damaged or exposed to harmful agents (Elmore, 2007). In *vitro* studies have shown an increased expression of the pro-apoptotic proteins Bax, Bad, and Bid following exposure to METH [6]. In the cellular process of apoptosis, the mitochondria play an important role (Chen et al., 2016). In addition, METH-induced mitochondrial damage may contribute to dopaminergic toxicity by increasing susceptibility to oxidative stress and promoting the apoptosis of neuronal cells [6]. The mechanisms of mitochondria-derived apoptosis triggered by METH require further investigation (Chen et al., 2016). In this study, the authors investigated the toxic potentials of METH in neuroblastoma cells and further

determined it effects on the mitochondrial activity.

2. METHODOLOGY

2.1 Cell Line and Cell Growth Media

The cell line that was used in this study were the SK-N-BE (2) which are the N-type neuroblastic cells, cell lines maintained in growth media. Growth media is important in cell culture to examine acell ina controlled environment.

The human neuroblastoma SK-N-BE (2) cells were cultured on Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Medium. The media DMEM/F12 is a1:1 mixture of DMEM and Ham's F-12. It is an extremely rich complex medium; DMEM/F12 supports the growth of a wide range of cell types to study their biological characteristics. According to the ATCC supplier of the SK-N-BE (2) (ATCC® CRL-2271™), it was recommended that the neuroblastoma cells be cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and incubated at 37° in a 5% CO_2 atmosphere.

2.2 Cell Thawing

The cryovialcontaining frozen SK-N-BE(2) cells in supplemented DMEM/F12 (95% FBS) and 5% Dimethyl-sulfoxide (DMSO) was removed from -80°C freezer and thawed in a 37°C water bath. The cryovial was decontaminated by wiping it with 70% ethanol and the contents of the vial transferred aseptically to a 15 mL conical centrifuge tube containing 2 mL of supplemented DMEM/F-12, centrifuged for 5 minutes at 1500 rpm. After centrifugation, the supernatant was discarded and the cell pellet re-suspended in 2 mL of supplemented medium. The cells were mixed thoroughly to ensure a homogeneous cell suspension, thereafter 1 mL was transferred to aT-25 culture flask. The transferred 1 mL of cells were combined together with 5 ml supplemented growth media in the T-25 flask. Cells were allowed to recover overnight in 37°C, 5% CO2 humidified incubator and were monitored microscopically for cell attachment. The media was discarded the next day, then replaced with fresh media and returned to incubator to allow for full recovery from resuscitation.

2.3 Cell Culturing

The flask containing the SK-N-BE (2) cells growth media was periodically changed until approximately 70-80% of confluency was reached. Cells were washed with warm 1XPBS (Phosphate Buffer Solution), thereafterthe1X PBS was aspirated. Then 2 mL of Trypsin was dispersed over the cell layer in flask and incubated for approximately 2-10 minutes for cell detachment. Thereafter immediately, 2 mL of supplemented media was added to the flask to deactivate the trypsin. The trypsinated cells were removed from flask, and transferred to a 15 mL conical centrifuge at 1500 rpm for 5 minutes. The cells were homogenized, then 1 mL was transferred to T-75 culture flask (surface area 75 cm2) containing10 mL supplemented media to maintain stock cultures and for use in experiments. The passage number was monitored at each sub culture event.

The method described by Redova et al. (2010) was adopted, where cells were sub-cultured on a 75cm2 flask and allowed to attach and reach confluency. Thereafter seeded onto a 6-well plate and allowed to attach for 24 hours before treatment, and untreated cells were used as a control. Cells were observed using an inverted microscope.

2.4 Cell Treatment with Methampheta-mine

Once the cell lines fully recovered, and reached confluency they were cryopreserved with 95% FBS + 5% DMSO (Dimethyl sulfoxide) and stored at-80ºC.

The SK-N-BE (2) cells were treated acutely with methamphetamine at different concentrations as follows: 1 µg/mL; 5 µg/mL; 10 µg/mL; 20 µg/mL; 50 µg/mL in 6-well plates, this treatment was administered once for 24 hours. At the end of 24 hours, culture medium with treatment was removed in the 48, 72, and 96 hour plates and replenished with culture media without methamphetamine treatment in order to maintain the acute environment required for the experiments. Two main factors were observed, which were dosage and duration. Which determined whether an increase in dosage concentration had an effect on the cells, as well as did the duration kept have an effect on the neuroblastoma cells.

2.5 Cell Viability Assay

Trypanblue is a negatively charged dye that stains a compromised cell membrane, which is indicative of cell death. Live cells have an intact

membrane, which prevents penetration of the cell membrane by the trypanblue dye. In dead cells, the dye passes through the permeable cellmembrane entering the cytoplasm (Fang & Trewyn, 2012). The cells aswell asblue coloured stainedcells were observed under light microscopy analysis. 50µL of cell suspension wasplaced in a cryo-vial, equalparts of 0.4% trypan blue dye was added to the cell suspension to obtain a 1:2 dilution and mix. Cell mixture was then incubated at room temperature for less than three minutes. One side of a hemocytometer counter was loaded with 20 µL of cell mixture. The hemocytometer was thenplaced on a light microscope to focus the cell, which enables counting of the cells for determination of viablecells. To obtain the total number of viable cells per ml of aliquot, the total number of viable cells will be multiplied by 2 (the dilution factor for trypanblue). To obtain the total number of cells per ml of aliquot, the total number of viable and nonviable cells were added up and multiplied by 2. The percentage of viable cells will be calculated asfollows:

$$Viable\ cells\ (\%) = \frac{\text{totalnumberofviablecellspermlofaliquot}}{Total\ number\ of\ cells\ per\ ml\ of\ aliquot} \times 100$$

2.6 Cell Proliferationcurve

The cell proliferative characteristics of then euroblastoma cell line were investigated by constructinga cell proliferation curve. Trypanblue dye was used for this purpose, to distinguish living cells (unstained) from dead cells (stained) which were not treated with methamphetamine once a cell countis performed microscopically. Viable cellcountswere averaged for construction of a SK-N-BE (2) cell proliferation curve. Throughout the experiment, the media was replenished every 24 hours to supply adequate nutrients to sustain cell growth. The experiment was repeated three times with a sample size of three (n =3).

The following equation was used for viable cell counts using trypan blue exclusion dyeto construct the growth curve:

$$Total\ viable\ cell\ count = total\ cell\ count \times \frac{Dilution\ Factor}{Number\ of\ square\ counted} \times 10^4$$

2.7 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT)Assay

The 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazoliumbromide (MTT) assays can be used to test for mitochondrial activity, in this study the XTT assay was used. The XTT assay is an effective method to measure cell growth and drug sensitivity in tumor celllines. XTT is a colorless orslightly yellow coloured compound that when reduced becomes brightlyorange.

2.8 XTT Preparation

To perform the XTT assay, a total cell count using trypanblue exclusion assay was conducted. Thereafter a density of 2×10^5 cells was required forseedingat respectivehours (24 hrs, 48 hrs, 72 hrs, and 96 hrs).

The XTT assay kit which includes a protocol was used to observe the mitochondrial activity and proliferation of the neuroblastomacells. According to the protocol manual from Thermo Fisher, 100 µL of the cells wasplaced using a pipette into the wells of a flat-bottom 96-well microtiterwell plate. Activated XTT reagent solution of 50 µL wasadded to the 100 µLcells. Theplates were then placed in a CO2 incubator for 4 hours, the plate was observed for an orange change in colour. The absorbance was measured in an Omega plate reader by BMG Labtechat a wavelength between 450 nm and 490 nm.

2.9 Statistical Analysis

Data were expressed as Mean ± Standard error of mean. One-way analysis of variance (ANOVA) and differences between means were determined using the Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA). The level of significance was set at $p<0.001$, 0.01 and 0.05.

3. RESULTS

3.1 Cell Proliferation Curve

SK-N-BE (2) cell growth rate was monitored over 4 days (24-96 hrs). Where 0 represents seeding hour on the day of cell seeding. The cells had a

slightly steady increase in cell number at 24 and 48hrs, thereafter entered an exponential growth phase between 48 to 96hrs. As proliferation progressed, 72 hrpresented with the greatest acceleration in exponential cell growth. This growth curve aids in the determination of seeding density used at the respective experimental hours for reproducibility to monitor the effect of methamphetamine treatment.

3.2 Total Cell Count

Following 24 hours of treatment with methamphetamine, the total cell count of the all the treated cells showed no statistical significant differences relative to the control (Fig. 2). After 48hours the total cell count showed a statistical significant decrease at the concentration of 10 µg/ mL (p=0.031) when compared to the control

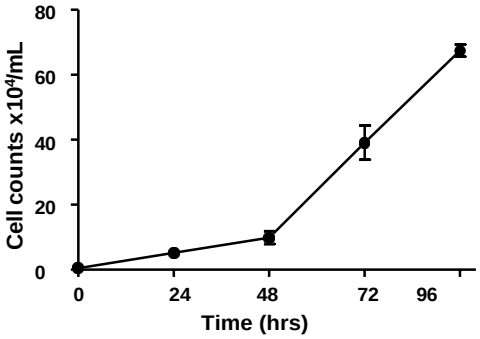

Fig. 1. Growth curve of untreated cells over 24 – 96 hours, monitoring the growth rate of SK-N-BE(2) cells

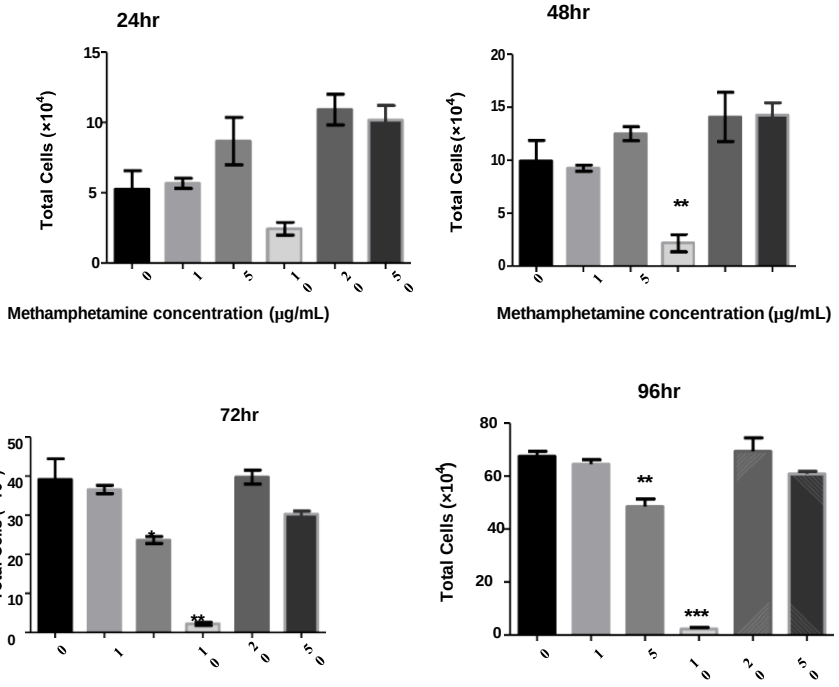

Fig. 2. Total count of SK-N-BE (2) cells acutely treated with varying concentrations of methamphetamine (1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL and 50 µg/mL) for 24 hours thereafter removed then replaced with culture media without methamphetamine treatment over a period of 96 hours. The data was collected intriplicates (n=3).The bars in the graph represent the mean and the error bars represent the standard error of mean (SEM). Statistically significant differences are marked by anasterisk (*) relative to the control which is represented by 0 and received no methamphetamine treatment. Statistically significant differences * indicates P value ≤ 0.05, ** indicates P value ≤ 0.01, and * indicates P value ≤0.001**

which received no methamphetamine treatment. After 72 hours the total cell count showed a statistical significant decrease at the concentration of 5µg/ mL (p=0.043) and at 10 µg/mL (p=0.007) when compared tothe control which received nomethamphetamine treatment. Lastly after 96 hours statistical significant decrease was observed at the concentration 5 µg/ml (p=0.004) and 10 µg/ml (p=0.000) when compared to the untreated control.

3.3 Cell Viability by Ratio of Live to Dead Cells Using Trypanblue Exclusionassay

SK-N-BE (2) cells treated with increasing concentrations of methamphetamine (1- 50 µg/mL) to observecell viability for 24 – 96 hours depicted in Fig. 3. No statistical significant difference in the means at 1 – 50 µg/mL as compared to the control was seen. Viability at prolonged or extended recovery period from methamphetamine exposure (48-96 hrs) showed a similar trend to those at 24 hours of which there was no effect in cellviability.

3.4 Toxicity by Ratio of Dead to Live Using Trypanblue Exclusionassay

There were no significant differencesin toxicity between treated cells ascompared tothe control at 24, 48, 72 and 96 hours. Toxicity amongst the control and cells treated with 10µg/ mLwas significantly increased (Fig. 4).

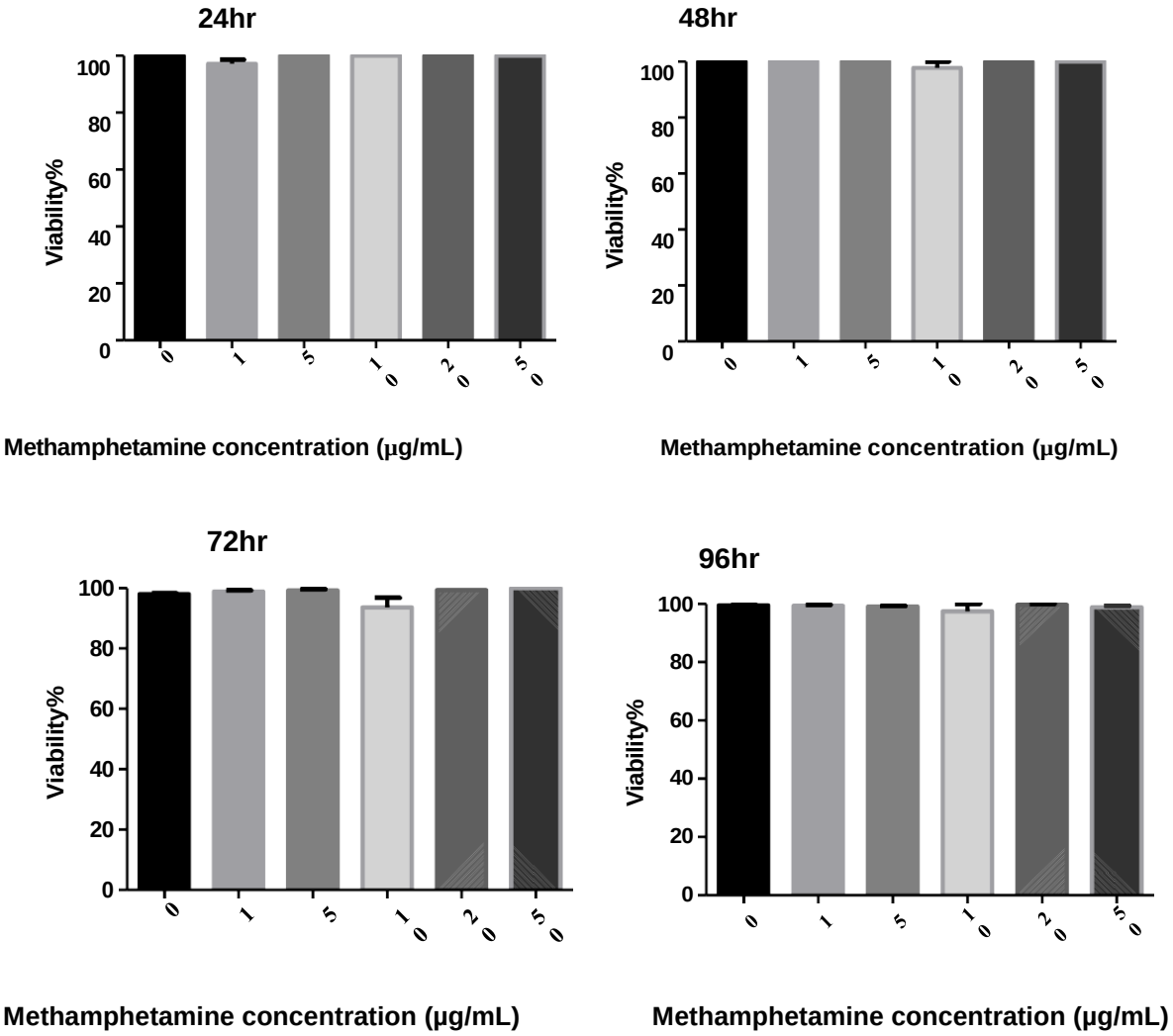

Fig. 3. Effect of methamphetamine treatment on cell viability after 24hr, 48hr, 72hr, and 96hr incubation period. Experiment was performed in triplicate (n=3). The bars in the graph represent the mean and the error bars represent the standard error of mean (SEM)

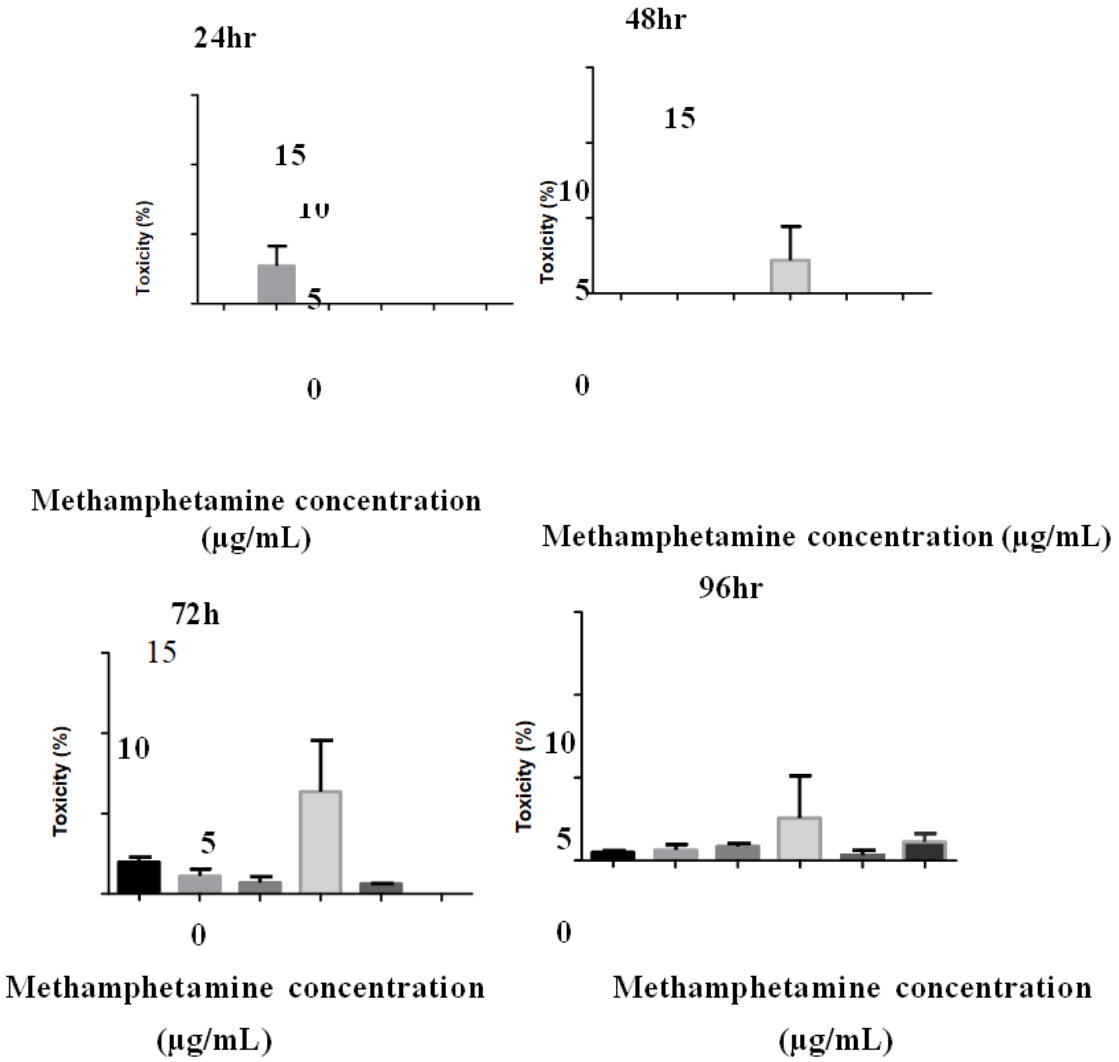

Fig. 4. SK-N-BE (2) cells acutely treated with different concentrations of methamphetamine over 96 hours. The data was collected in triplicates (n=3). The bars in the graph represent the mean and the error bars represent the standard error of mean (SEM) [Mean ± SEM]

3.5 Comparison of Live to Dead Cells Using Trypanblue Exclusion assay

To test the cell viability on the SK-N-BE (2) cells in this study the Trypanblue exclusion assay was used.

3.6 Mitochondrial Activity

Following treatment with methamphetamine, the mitochondrial activity of the all the treated cells showed significant differences relative to the control (Fig. 6). After 24 hours mitochondrial activity represented by absorbance showed a significant decrease at the concentration of 10 µg/mL (p=0.027) when compared to the control which received no methamphetamine treatment. After 48 hours the absorbance decreased significantly at the concentration of 10 µg/mL (p=0.001) when compared to the control which received no methamphetamine treatment. After 72 hours the absorbance decreased significantly at the concentrations 5 µg/mL (p=0.000); 10 µg/mL (p=0.000); 20 µg/mL (p=0.000); and 50 µg/mL (p=0.000). Lastly after 96 hours significant decrease was observed at the concentration 10 µg/ml (p=0.001) when compared to the untreated control.

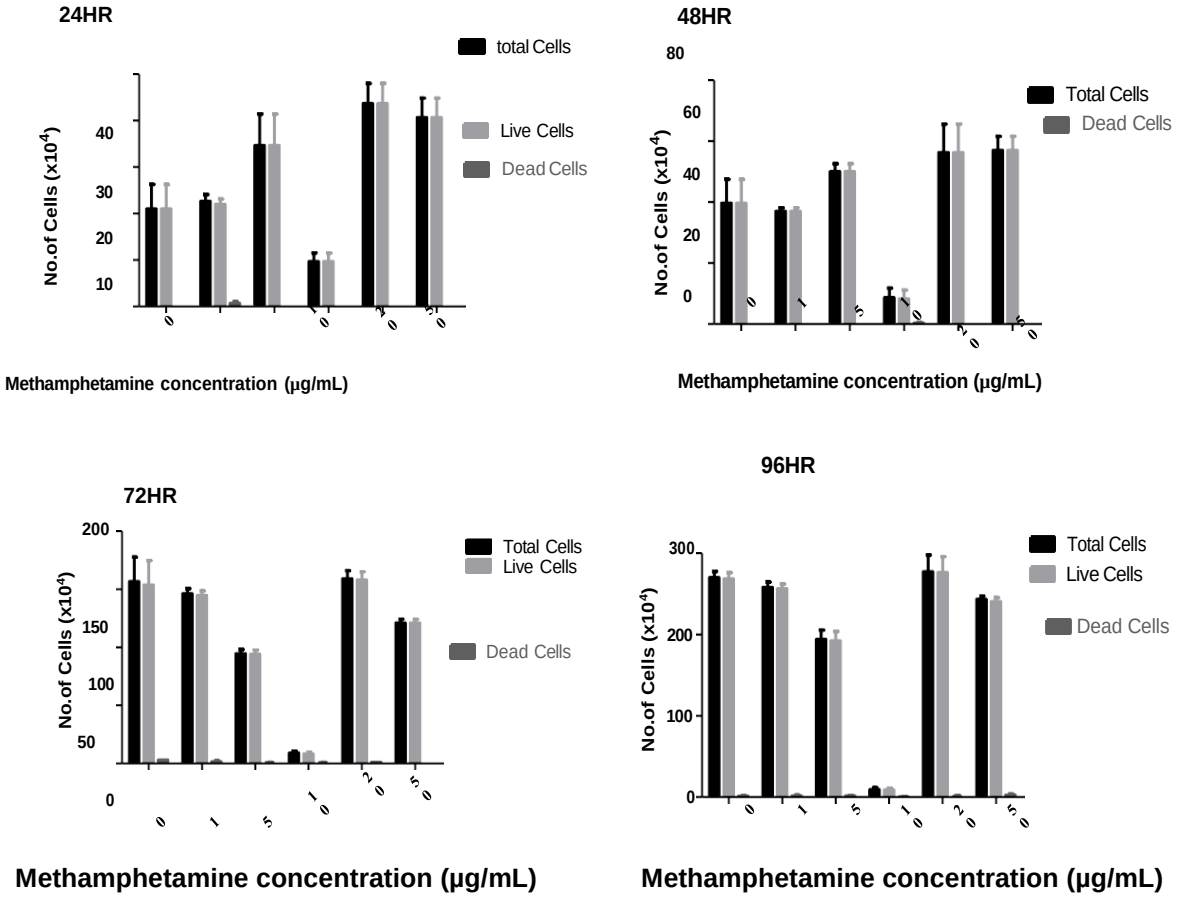

Fig. 5. Comparison of total number of cells, to live and dead cells using trypan blue exclusion assay

4. DISCUSSION

Cell growth observed at the time intervals experimented in this study showed that the cells conformed to normal cancer growth and division. According to ATCC the population, doubling time of the neuroblastoma cell line is 18 hours, and this was illustrated by the cell proliferation curve in Fig. 1.

The considerable decrease in the SK-N-BE (2) cells following acute treatment with10µg/mL METH for 48, 72, and 96 hours, may be attributed to the negative effect on cellular signals that induce growth. Cell proliferation is maintained by a strict coordination of cellular signals and deregulation of cell proliferation is the defining feature of all tumors [13]. Cancer progression is controlled by eight critical biological processes and these include the ability: to sustain proliferative signals, to evade growth-suppressors, to invade and metastasize, to enable replicative immortality, to induce angiogenesis, to resist cell death, to escape immune destruction, and to deregulate cellular metabolism [14]. The observed decline in cell number recorded in this study might be because METH disturbs one or more of these biological processes. Furthermore, a study conducted by Jackson et al. [15] on astrocytes, showed that exposure of the cells to METH affected the proliferation of the cells on cell cycle progression. Thus can be suggested that METH is neurotoxic after acute treatment at the concentration of 10 µg/mL. Result illustrating the number of dead and live cell further substantiates this observation at 10 µg/mL acute treatment. Conversely, at higher concentration (20 and 50 µg/mL) where there is little to no dead cells, it could be suggested that METH slightly induced cell proliferation, since the controlled cells are fewer than the cells treated with 20 µg/mL of METH after 48, 72, and 96hours. This observation is supported by previous submission. Moszcznska et al. [16]; Moszcznska and Callan, [17] stated that high doses of METH induce DNA breaks which leads to induction of mobile genes (transposons-LINE)

and its integration with the DNA. This can increase proliferation and survival of the cells. LINE is highly mutagenic and associated with multiple steps in cancer. LINE encodes proteins that drive oncogenic mechanisms and hypomethylation process. High dose of METH hence allows cancer progression. On the other hand, this was contradicted by former authors as Jacksons et al. (2014) who reported that methamphitamine slightly increased the number of the cells entering G1 phase of the cycle but decreases the number of cells that progress through G2-S-phase due to down regulation of genes involved in cell cycle progression.

In addition, cell viability exceeding 95% after 24hours acute treatment and toxicity score below 5 % at 24, 48, 72, and 96 hours acute treatment suggested that methamphetamine promotes the growth of cells. ROS productions are potent initiator of cell death. as suggested by Badisa et al. [18], lack of cell death following acute METH treatment infer lack of ROS production and nitric oxide generation or cell cycle inhibition [18]. Only the viability of cells treated with 10 µg/mL after 48 hours, was below the benchmark, suggesting that METH has a neurotoxic effect that affects viability since 93% suggests that cells are not well viable.

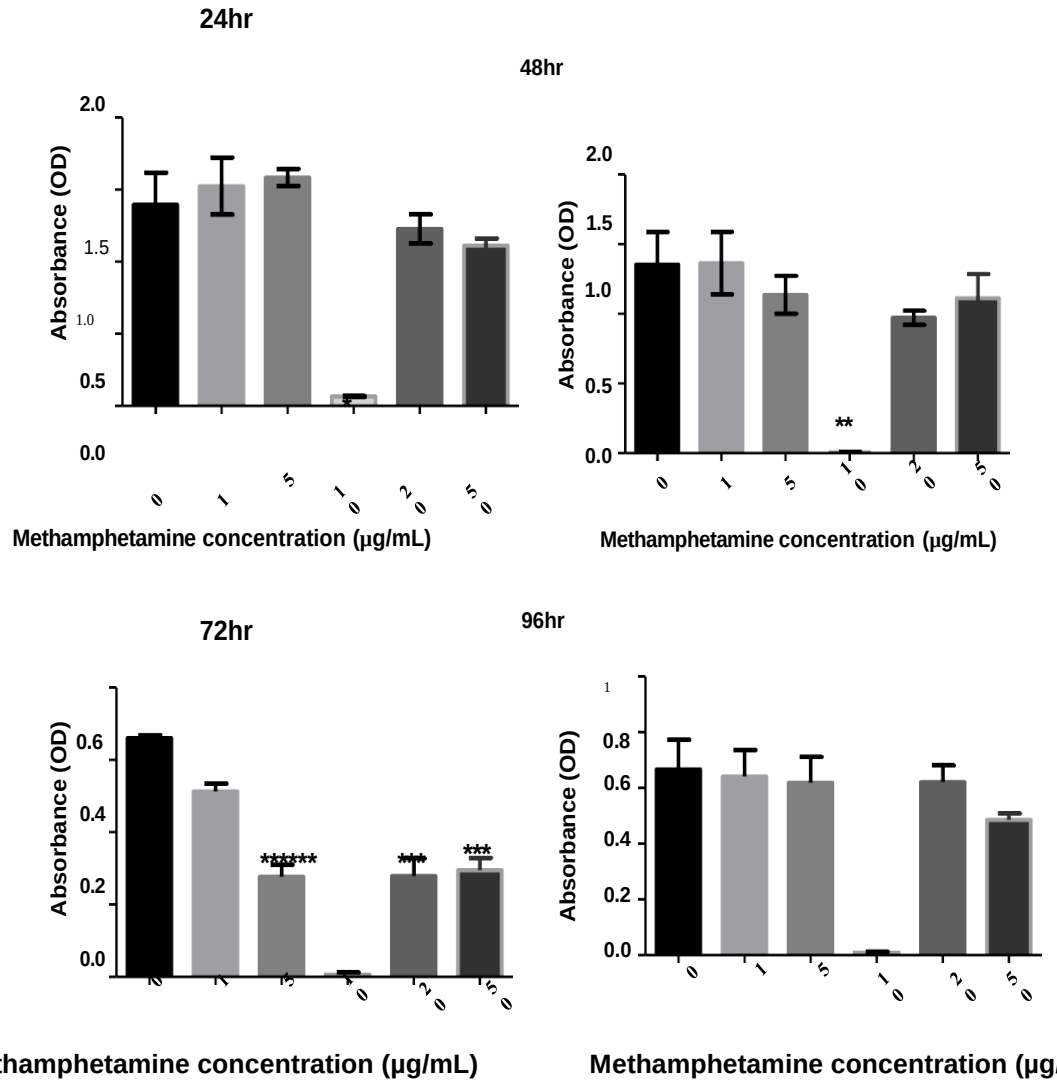

Fig. 6. Mitochondrial activity represented as optical density value of SK-N-BE (2) cells acutely treated with varying concentrations of methamphetamine (1µg/mL, 5µg/mL, 10µg/mL, 20µg/mL and 50 µg/mL) over 24 - 96 hours. The data was collected intriplicates (n=3) there after averaged. The barsre present the mean and the error bars represent the standard error of mean (SEM). Statistically significant differences are marked by an asterisk (*), * indicates P value ≤ 0.05, ** indicates P value ≤ 0.01, and * indicates P value ≤0.001**

Maintenance of the mitochondrial membrane potential is important for oxidative phosphorylation activity and therefore any decrease in mitochondrial membrane potential will result in mitochondrial dysfunction [19]. A number of studies have documented that METH can cause neuronal apoptosis through cross-talks between the mitochondria, endoplasmic reticulum, and receptor mediated death pathways [19]. The administration of METH can result in disruption of mitochondria via both direct and indirect mechanisms, METH is a cationic lipophilic molecule that can diffuse the mitochondria and be retained there [20]. Methamphetamine is known to trigger the production of reactive oxygen species, which causes damage within the cell. The production of the reactive species occur through three mechanisms namely, dopamine release and subsequent enzymatic oxidation; dopamine auto-oxidation; and mitochondria disruption [20]. Which ultimately results in METH-induced neurotoxicity.

The decline in mitochondrial activity observed when the SK-N-BE (2) cells were treated with METH at all thetime intervals is indicative of damage to mitochondrial function, which contributed to the failure of the mitochondrial activity of the cells.

5. CONCLUSION

The result of this study revealed that METH altered the growth of SK-N-BE (2) cells following acute treatment. At 10 µg/mL, proliferation of the cells was inhibited whereas at concentration above, it might slightly induce cell proliferation. Compromise in the cell viability at 10 µg/mL might be associated with oxygen radical production, which is a known initiators of cell death. Finally, the mechanism behind METH induced neurotoxicity might be associated with inhibition of mitochondrial activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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