Methadone hydrochloride and leukemia cells: Effects on cell viability, DNA fragmentation and apoptotic proteins expression level

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Abstract: Leukemia is a type of blood cancer where abnormal and immature leucocytes are produced in the bone marrow. Methadone hydrochloride is a man-made drug that is commonly used in the maintenance therapy for drug addiction. The objective of this research was to determine the cytotoxic activity and apoptosis induction capability of methadone hydrochloride towards two leukemia cell lines which are CCRF-CEM and HL-60. Treatment of these cell lines was done with methadone hydrochloride for 24 and 48 hours to determine the cytotoxic activity. IC50 at 24 hours obtained for CCRF-CEM is 121.6µmol/L while IC50 for HL-60 cells is 97.18 µmol/L. Result obtained from DNA fragmentation assay showed no characteristic DNA ladder pattern in CCRF-CEM leukemia cells treated with methadone hydrochloride. Characteristics DNA ladder pattern was observed in methadone hydrochloride treated HL-60 cells. Formation of comets was seen in methadone hydrochloride treated leukemia cells with varying degree of DNA damage. The comets formed by methadone hydrochloride treated HL-60 cells were more prominent as compared to methadone-treated CCRF-CEM cells. The level of apoptosis-related proteins in methadone-treated CCRF-CEM and HL-60 leukemia cells were checked by incubating the cell lysate with Raybio® Human Apoptosis Antibody Array. Significant alterations in the level of apoptosis-associated proteins in methadone hydrochloride treated CCRF-CEM cells were found involving upregulation of Caspase 8 expression and downregulation of survivin expression. Methadone hydrochloride triggered apoptosis in HL-60 cells involved upregulation of Bid and Caspase 8 expression along with downregulation of Bcl-2, p21, and survivin expression.

Keywords: Methadone hydrochloride, apoptosis, leukemia.

INTRODUCTION

Leukemia, cancer of blood forming cells, can either be acute or chronic (Yazdian-Robati *et al*., 2017). There are four major kinds of leukemia which are the acute lymphocytic, chronic lymphocytic, acute myelocytic and chronic myelocytic leukemia. It is the most commonly found cancer in children whereas acute lymphocytic leukemia is the commonly diagnosed leukemia while the remaining cases are mostly of acute myeloid leukemia (Han *et al*., 2017).

Methadone is a synthetic opioid that existed more than 70 years ago and is commonly used for maintenance treatment for heroin addicts (Dole and Nyswander, 1967). Methadone is available as the hydrochloride salt. It is a combination of stereoisomers which are L-methadone and D-methadone (Trescot *et al*., 2008). A research done by Friesen *et al*. (2008) showed that methadone hydrochloride kill leukemia cells through apoptosis and it even has the ability to kill leukemia cells that are doxorubicin resistance, multidrug resistance and apoptosis resistance without affecting the peripheral blood lymphocytes (Friesen *et al*., 2008).

Apoptosis is a genetically regulated process which serve as a function to eliminate excessive or damaged cells in an organism (Kam and Ferch, 2000; Khan *et al*., 2014; Goldar *et al*., 2015). To date, there are 2 mainly reported apoptotic pathways: (i) the extrinsic pathway, and (ii) the intrinsic pathway (Elmore, 2007; Derakhshan *et al*., 2017).

Initiation of intrinsic pathway involves various stimuli including radiation, hypoxia, toxins, DNA damage, the absence of hormones, and certain cytokines to suppress apoptosis and does not involve binding of death receptor and its ligand (Elmore, 2007). Signals activated by these stimuli will increase mitochondrial membrane permeabilization which are regulated through the Bcl-2 family members (Schug *et al*., 2011). Bcl-2 family pro-apoptotic proteins are Bax, Bok, Bak, Bim, Bad, NOXA, Bid and PUMA. Anti-apoptotic proteins consist of Bcl-xL and Mcl-1. Two major groups of proteins were discharged into the cytosol. The very first group of proteins consists of Smac/DIABLO, cytochrome c, and serine protease HtrA2/Omi. Binding of cytochrome c together with procaspase 9 and Apaf 1 will form apoptosome that will lead to activation of caspase 9 (Elmore, 2007; Kantari and Walczak, 2011).

Extrinsic apoptotic pathway proceeds through binding of death receptor and its ligand (Elmore, 2007). There are

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two different pathways for an extrinsic pathway to proceed with which is Type I or Type II (Parrish *et al*., 2013). Caspase 8 activation and the degree of production of death-inducing signaling complex (DISC) are important to determine whether extrinsic pathway will proceed with either Type I or Type II (Schug *et al*., 2011). In Type I, direct cleavage of effector caspases is possible due to the formation of a significant amount of DISC and active Caspase 8 (Lee *et al*., 2006; Schug *et al*., 2011). However, in Type II, engagement to the intrinsic pathway via Caspase 8 is required for signal amplification and execution of apoptosis due to a slower assembly of DISC and low yield of active Caspase 8 (Peter and Krammer, 2003). Bid cleavage to truncated Bid (tBid) through Caspase 8, is required to change extrinsic pathway to intrinsic pathway of apoptosis (Kantari and Walczak, 2011). Interaction of tBid and Bcl-2 family proteins on surface of mitochondrial will result in mitochondrial outer membrane permeabilizations (Schug *et al*., 2011).

In this study, we have explored the cytotoxic activity and apoptotic effects of methadone hydrochloride treatment towards two leukemia cell lines which are CCRF-CEM and HL-60 through MTS assay, DNA fragmentation assay, and Raybio® Human Apoptosis Antibody Array.

MATERIALS AND METHODS

Cell lines

HL-60 and CCRF-CEM human leukemia cell lines were purchased from American Type Culture Collection. CCRF-CEM cells (ATCC® CCL-119TM) were cultured in RPMI-1640 medium having 10% FBS while HL-60 cells (ATCC® CCL-240TM) were cultured in Iscove's Modified Dulbecco's Medium supplemented with 20 percent FBS. Culturing of these two cell lines was done at 37°C under a 5 percent CO_2 humidified atmosphere.

Chemicals and reagents

All the reagents and chemicals were obtained from the indicated companies: RPMI-1640 media, IMDM, FBS, Penicillin/streptomycin (Gibco, US); Methadone hydrochloride powder (Lipomed AG, Switzerland); CellTiter 96® AQ_{ueous} One Solution Cell Proliferation OxiSelect™ Comet Assay kit (Cell Biolabs, Inc., CA); Raybio® G-Series Human Apoptosis Antibody Array (RayBiotech, US); Phosphate Buffer Saline (PBS) tablets (Invitrogen, USA).

Methadone hydrochloride stock solution was prepared freshly prior to use by dissolving the powder with sterile distilled water and filtered through 0.22 µm syringe filter.

Assay for cytotoxic activity

CellTiter 96® AQueous One Solution Cell Proliferation Assay having MTS was used to determine the in-vitro cytotoxic activity of methadone hydrochloride towards two leukemia cell lines. Briefly, cells were seeded in 96-well plate and in duplicate plates (24 and 48 hours), with triplicate wells (2 x 10^4 cells in each well) in 100µL of the culture media. Three controls of medium alone which serve as blank for absorbance readings were included. Seeded plates were then placed overnight at 37°C in the incubator. 10µL of increasing concentration of methadone hydrochloride was added into the assigned wells. 20µL of CellTiter reagent was added into each well one by one and then incubation was done for further 4 hours. Absorbance was documented at 490nm using Elisa plate reader. The absorbance of untreated cells was taken as control (100 percent viability) and background absorbance (mean absorbance of blank) was subtracted. The formula utilized for the calculation of the percentage of cancer cell viability was:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance sample} - \text{Absorbance blank}}{\text{Absorbance control} - \text{Absorbance blank}} \times 100$$

DNA fragmentation assay

Quick Apoptotic DNA Ladder Detection kit was used to evaluate the level of DNA fragmentation in two leukaemia cells after treatment with methadone hydrochloride following manufacturer's protocol. The CCRF-CEM and HL-60 cell lines were treated with their IC_{50} value and two times of their IC_{50} value of methadone hydrochloride concentration. Both of the cell lines were then treated with apoptosis inducer as a positive control and another set of cells was left untreated as a negative control. 5 – 10 x 10^5 cells were pelleted and washing was done with PBS. The degree of DNA fragmentation induced via methadone hydrochloride was analyzed by using 1.2 percent agarose gel supplemented with ethidium bromide at a concentration of 0.5 µg/mL (100 V, 1 hour) visualized by trans illumination with ultraviolet light.

Comet assay

Oxi Select TM Comet Assay kit was used to check DNA damage stimulated by methadone hydrochloride towards CCRF-CEM and HL-60 cells at a single cell level. Cells were plated and treated with methadone hydrochloride using their respective IC_{50} values. A small quantity of cells were suspended in thin agarose gel in a well on a treated slide before being lysed, electrophoresed as well as stained with the DNA staining dye. Fluorescence microscopy was used to view the cells on the slide. With the help of Casplab_1.2.3b2 software, DNA damage on cells was analyzed and quantified at an individual level.

Apoptosis antibody array

Human Apoptosis Antibody Array was used to study the efficacy of methadone hydrochloride on the level of 43 proteins in two human leukemia cell lines, following the manufacturer's protocol. These two cell lines were cultured and treated in 6-well plates with their respective IC50 values obtained from the cell cytotoxicity assay for 24 hours. The cell lysate was obtained by lysing the cells

with cell lysis buffer provided followed by a collection of the supernatant after centrifugation. The array was incubated with the treated cell lysate, followed by incubation with biotinylated antibody and lastly labeled streptavidin. The array slide was scanned by Genomax Company using a laser scanner. The signal intensities obtained were converted into readable data, and with the help of the analysis software, the expression level of each protein is obtained.

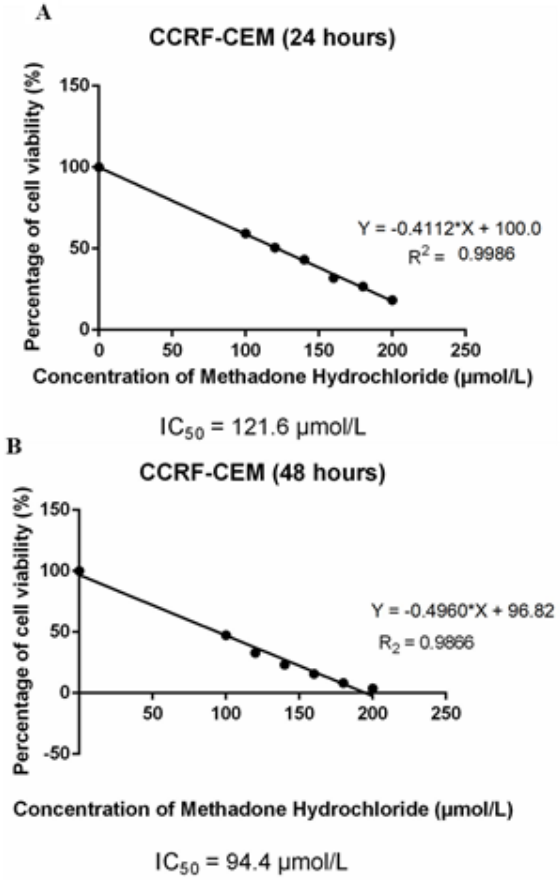

Fig. 1: Cell viability assay on CCRF-CEM cells using methadone hydrochloride. CCRF-CEM cells were treated with increasing concentration of methadone hydrochloride for (A) 24 hours and (B) 48 hours.

RESULTS

Cytotoxic activity of methadone hydrochloride against CCRF-CEM and HL-60 cells

Data shown in Figure 1 was obtained after 24 hours and 48 hours of methadone hydrochloride treatment. 121.6 µmol/L and 94.4 µmol/L concentrations of methadone hydrochloride respectively were needed to kill the CCRF-CEM cells by half. Meanwhile, as shown by the graph in Figure 2, for HL-60 cells, IC_{50} for 24 hours and 48 hours treatment of methadone hydrochloride were 97.18µmol/L and 87.8µmol/L respectively. Result obtained through Figure 1 and Figure 2 display that methadone hydrochloride treatment repressed cell proliferation in a time and dose-dependent way in these two leukemia cell lines.

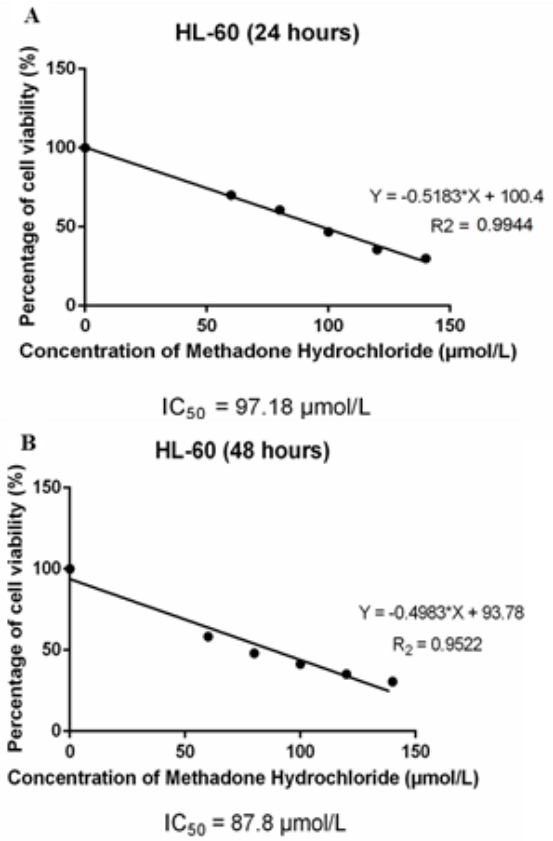

Fig. 2: Cell viability assay on HL-60 cells using methadone hydrochloride. HL-60 cells were treated with increasing concentration of methadone hydrochloride for (A) 24 hours and (B) 48 hours.

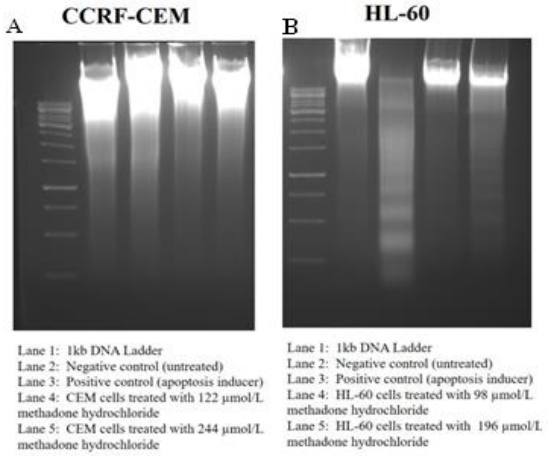

Fig. 3: DNA fragmentation in leukemia cells treated with methadone hydrochloride. CCRF-CEM cells (A) and HL-60 cells (B) were given treatment with their respective IC_{50} value and two times of their IC_{50} value for 24 hours before their DNA being extracted and run on 1.2 % agarose gel. Characteristics DNA ladder pattern was observed in methadone hydrochloride treated HL-60 cells (B) while no characteristics DNA ladder pattern was

observed for methadone hydrochloride treated CCRF-CEM cells (A).

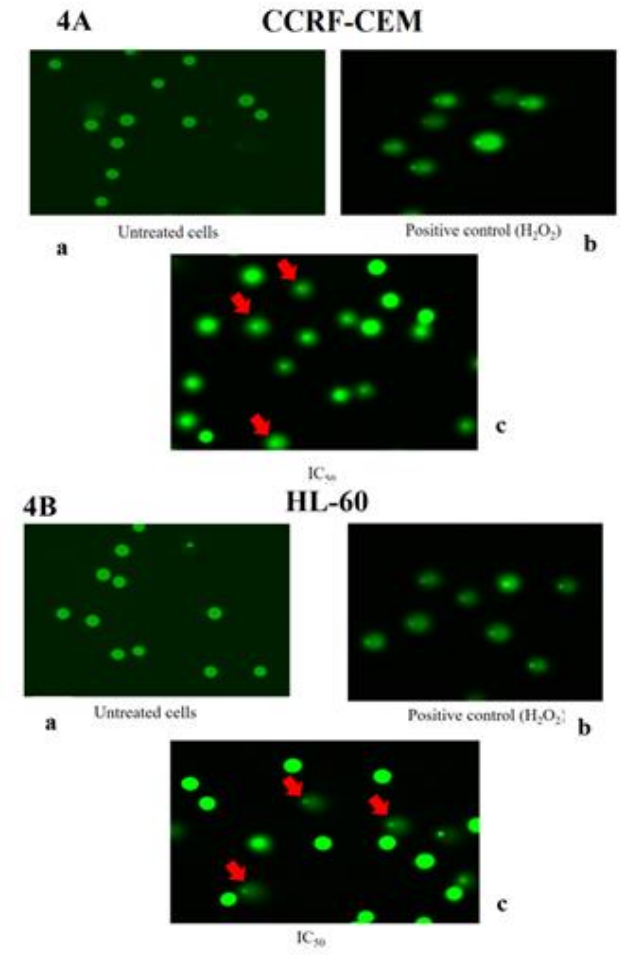

Fig. 4(A): CCRF-CEM cells stained with Vista Green DNA Dye under a fluorescence microscope. (a) CCRF-CEM cells without methadone hydrochloride treatment served as control. (b) CCRF-CEM cells treated with H_2O_2 to induce apoptosis were utilized as positive control. (c) CCRF-CEM cells treated with methadone hydrochloride (121.6 µmol/L) for 24 hours, 4(B). HL-60 cells stained with Vista Green DNA Dye under a fluorescence microscope. (a) HL-60 cells without methadone hydrochloride treatment to serve as a negative control. (b) HL-60 cells treated with H2O2 to induce apoptosis as a positive control. (c) HL-60 cells with treatment of methadone hydrochloride (97.18 µmol/L) for 24 hours.

Methadone hydrochloride induced DNA fragmentation in HL-60 cells

Based on the results shown in Figure 3(A), no characteristic DNA ladder pattern was observed in CCRF-CEM leukemia cells treated with methadone hydrochloride. While Figure 3(B) displayed characteristic DNA ladder pattern in the agarose gel loaded with the extracted DNA from methadone hydrochloride treated HL-60 leukemia cells. DNA ladder pattern is more prominent in cells treated with twice times IC_{50} value comparing to the cells treated with IC_{50} value.

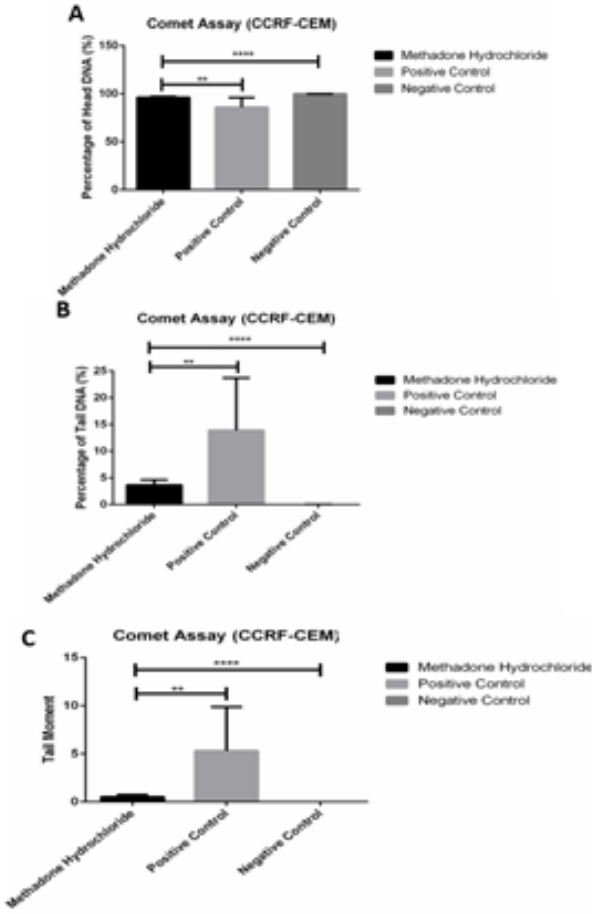

Fig. 5: Percentage of Head DNA, Tail DNA and Tail Moment for CCRF-CEM cells (negative control, positive control, and treatment with methadone hydrochloride) for 24 hours (mean ± SD, n=10) using comet assay. The Tail DNA percentage for negative control, positive control, and methadone-treated CCRF-CEM are 0.006%, 13.904%, and 3.668% respectively.

Methadone hydrochloride treatment induced DNA damage in leukemia cells

Figure 4A showed pictures of CCRF-CEM cells (untreated, treated with H_2O_2 and treated with methadone hydrochloride for 24 hours) stained with Vista Green DNA Dye viewed under a fluorescence microscope.

Percentage of the head and tail DNA for CCRF-CEM cells (negative control, positive control, and treatment with methadone hydrochloride) for 24 hours was shown in fig. 5.

Fig. 4B showed pictures of HL-60 cells (untreated, treated with H_2O_2 and treated with methadone hydrochloride for 24 hours) stained with Vista Green DNA Dye viewed under a fluorescence microscope.

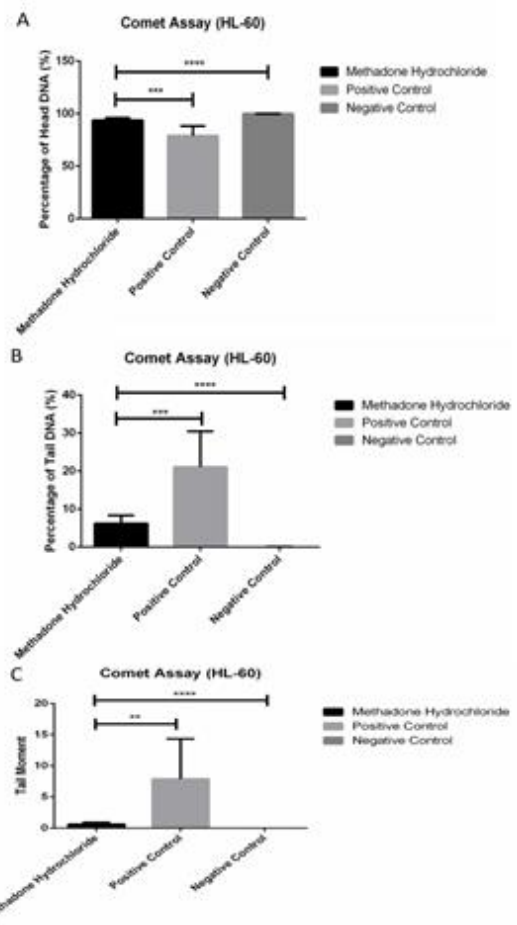

Fig. 6: Percentage of Head DNA, Tail DNA and Tail Moment for HL-60 cells (negative control, positive control, and treatment with methadone hydrochloride) for 24 hours (mean ± SD, n=10) using comet assay. The Tail DNA percentage for negative control, positive control, and methadone-treated HL-60 are 0.019%, 21.071%, and 6.202% respectively.

Percentage of the head and tail DNA for HL-60 cells (negative control, positive control, and treatment with methadone hydrochloride) for 24 hours was shown in fig. 6.

The error bars in the chart represent the standard deviation. Statistical significance was evaluated through unpaired t-test. A calculated p-value of ≤ 0.05, ≤ 0.01, ≤ 0.001 and ≤ 0.0001 was deemed a significant difference and indicated with a single asterisk (*), double asterisk (**), triple asterisk (***) and quadruple asterisk (****) respectively.

Methadone Hydrochloride Down-regulated the Expression of Bcl-2, Bid, p21, and Survivin while Up-regulated the Expression of Caspase-8

The apoptosis-related proteins with noticeable changes in expression level for methadone-treated HL-60 cell lysate, comparing to the untreated control as shown in Figure 7 (A) are Bcl-2, bid, caspase 8, p21, and survivin.

As shown in fig. 7 (B), apoptosis-related proteins with noticeable changes in expression level for methadone-treated CCRF-CEM cell lysate, comparing to the untreated control are Caspase 8, p53 and survivin.

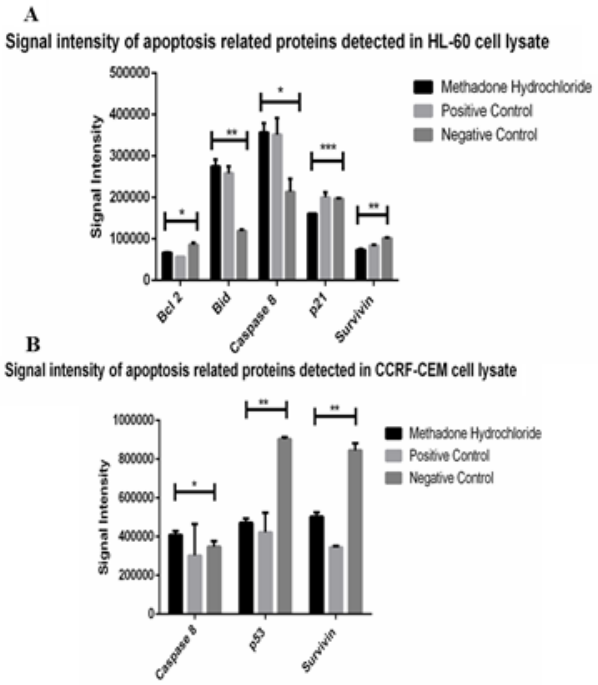

Fig. 7(A): The expression level of apoptotis-affiliated proteins in HL-60 cells which were given treatment of methadone hydrochloride. HL-60 cells were treated with its IC_{50} value for 24 hours before being lysed and incubated with the array. (B) The expression level of apoptosis-associated proteins in CCRF-CEM cells treated with methadone hydrochloride. CCRF-CEM cells were treated with its IC_{50} value for 24 hours before being lysed and incubated with the array.

The error bars in the chart represent the standard deviation. Unpaired t-test was used for the determination of statistical significance. A calculated p-value of ≤ 0.05, ≤ 0.01, ≤ 0.001 and ≤ 0.0001 was deemed a significant difference and indicated with a single asterisk (*), double asterisk (**), triple asterisk (***) and quadruple asterisk (****) respectively.

DISCUSSION

Methadone hydrochloride, a long-acting μ opioid receptor agonist, is used in the maintenance therapy for opioid addiction (Brawanski *et al*., 2018). Methadone hydrochloride is also used as an analgesic in the cure of mild to severe cancer and neuropathic pains (Porta-Sales *et al*., 2016; McNicol *et al*., 2017). Leukemia cells have been reported to express μ opioid receptor (Beltran *et al*., 2006). Previous studies revealed the capability of methadone hydrochloride to trigger apoptosis in leukemia cells along with human lung cancerous cells (Friesen *et al*., 2008). Anticancer drugs function via activation of

apoptosis by extrinsic or mitochondrial pathways (Baig *et al*., 2016). Previous investigations have documented that methadone hydrochloride induce apoptosis in a same manner as currently available anti-cancer drugs (Friesen *et al*., 2008). Increased knowledge of how methadone hydrochloride stimulated apoptosis in leukemia cells is critical in understanding the mechanism involved in drug-induced apoptosis for the discovery of new drugs for effective medication of leukemia. In our study, we have determined the anti-cancer potential of methadone hydrochloride on two leukemia cells CCRF-CEM and HL-60 and delineated the mechanism of methadone hydrochloride-induced apoptosis in these cancer cells.

Results obtained from MTS assay showed that methadone hydrochloride inhibited proliferation of CCRF-CEM and HL-60 leukemia cells in a concentration-dependent mode. No characteristic DNA ladder pattern was found in CCRF-CEM leukemia cells which were treated with methadone hydrochloride suggesting that apoptosis is not induced in methadone hydrochloride treated CCRF-CEM cells. However, the absence of DNA ladder cannot indicate apoptosis induced as DNA fragmentation appears in the next phase of apoptosis. Therefore, cells may be undergoing early apoptosis (Elmore, 2007). In addition to that, DNA fragmentation does not necessarily present in cells that are undergoing apoptosis. This is because a certain type of cells shows morphological and biochemical features of apoptosis without the characteristics DNA ladder pattern (Edebali *et al*., 2014). While in case of HL-60 cells, DNA ladder pattern is more prominent in cells treated with two times IC50 value comparing to the cells treated with just IC50 value suggesting that the degree of apoptosis is dose-dependent. Formation of comets was also seen in methadone hydrochloride treated CCRF-CEM and HL-60 cells with varying degree of DNA damage.

Previous study reported that methadone hydrochloride treatment induced apoptosis via intrinsic pathway in leukemia cells which involved caspase 9/3 activation, reduction in the level of Bcl-XL (Friesen *et al*., 2008). However, in our study, we found out that methadone hydrochloride mediated apoptosis in CCRF-CEM cells and HL-60 cells through activation of extrinsic and intrinsic pathway respectively.

Apoptosis proceeds mainly by two pathways in cells, Extrinsic and Intrinsic. Caspase 8 is a member of cysteine protease family that plays a significant role in extrinsic pathway of apoptosis (Pfeffer and Singh, 2018). Upregulated expression of Caspase 8 in methadone hydrochloride treated CCRF-CEM cell lysate suggests that methadone hydrochloride induced apoptosis in CCRF-CEM cells via extrinsic pathway.

Further we have explored that whether intrinsic pathways was also involved in methadone hydrochloride induced apoptosis. Bcl-2 is one of the members of Bcl-2 family that is involved in intrinsic apoptotic pathway. In cancerous cells, anti-apoptotic proteins like Bcl-2 family are frequently upregulated to evade apoptosis (Adams and Cory, 2018). Methadone hydrochloride stimulated apoptosis in HL-60 cells via downregulating Bcl-2.

p53 is a tumor suppressor protein where its activation through internal and external stress signals will lead to nuclear aggregation of active form p53 leading to apoptosis, senescence or arrest of cell growth (Zhang *et al*., 2017). So, further we want to investigate that whether apoptosis induced was dependent or independent of p53.The expression level of p53 for methadone-treated cell lysate was down-regulated compared to untreated cell lysate of CCRF-CEM. The result showed that methadone hydrochloride promoted apoptosis in cancer cells was independent of p53.

p21, a downstream target of p53, perform principle role in cell cycle arrest by blocking the function of cyclin dependent kinases. However, p21 has dual role in a cell it can be a cell cycle arrest inducer or anti-apoptotic factor. How it will function it depends upon where it is localized. In nucleus, activated p21 function as cell cycle arrest inducer while if in cytoplasm, a lower level of p21 induce apoptosis (Liu *et al*., 2003; Abbas and Dutta, 2009). Methadone hydrochloride treated HL-60 cells express down-regulated expression of p21. It suggests that p21 might be localized in cytoplasm in HL-60 cells where its down-regulation has induced apoptosis.

Survivin is a well-known member of apoptosis inhibitor protein family that inhibits apoptosis (Mita *et al*., 2008). A lower expression of survivin directly activates caspase 3 which ultimately leads cells towards apoptosis (Tan *et al*., 2009). Downregulated expression of survivin was detected in methadone-treated CCRF-CEM cell lysate which suggest that inhibition of survivin along with Bcl-2 might show more effective apoptotic effects.

CONCLUSION

In conclusion, the current study shows that methadone hydrochloride inhibited proliferation of CCRF-CEM and HL-60 leukemia cells in a concentration-dependent mode. The anti-cancer mechanism of action of methadone hydrochloride proceeds via inducing apoptosis in CCRF-CEM cells and HL-60 cells through extrinsic and intrinsic pathways. Up-regulated expression of Caspase 8 in methadone hydrochloride treated CCRF-CEM cell lysate suggests that methadone hydrochloride induced apoptosis in CCRF-CEM cells via extrinsic pathway while down-regulation of Bcl-2 expression suggests induction of intrinsic apoptosis. Moreover, our results demonstrated that methadone hydrochloride induced apoptosis was independent of p53, however, it downregulated the expression of p21. Taken together, our results recommend

that methadone hydrochloride induced cytotoxicity and apoptosis in CCRF-CEM and HL-60 cells, which provides the rationale for future *in vitro* and *in vivo* studies on the utilization of methadone hydrochloride as an anti-cancer agent.

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