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Attenuation of Morphine-Induced Camp Overshoot by Thymoquinone in Opioid Receptor Expressing Cells (U87 MG) Mediated by Chronic Morphine Treatment

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Abstract: Chronic opioid intake leads to opioid dependence and withdrawal syndrome after opioid cessation. Morphine and Thymoquinone (TQ) are both opioid receptor stimulating compounds, although, they have different pharmacological origins. Morphine is a natural opioid derivative whereas TQ is one of the main pharmacologically active compounds from *Nigella sativa* oils. This study was carried out to study the effects of TQ on cAMP concentration mediated by chronic morphine treatment in opioid receptor expressing cell line (U87 glioblastoma cells). U87 cells was grown in tissue culture flasks with RPMI 1640 medium containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (w/v) penicillin/streptomycin. The cell viability was assessed by the trypan blue dye and manually counted using a haemocytometer. The MTT assay was used to determine the cytotoxic effects of morphine and TQ. The cAMP concentration in the cells was determined using the Cell Biolabs, Inc. cAMP ELISA Kit. Co-treatment of morphine and TQ significantly attenuate the increase in cAMP content produced by morphine after chronic morphine treatment (*$p<0.05$). These finding suggest that TQ could possibly reduce opioid dependence on chronic morphine treatment at cellular level by reducing the up-regulation of cAMP level.

Key words: Morphine, thymoquinone, cAMP concentration, opioid receptor expressing cell (U87 MG), up-regulation, TQ

INTRODUCTION

Opioid addiction is a serious brain disease that often associates with opioid dependence and withdrawal syndromes. Abrupt cessation of chronic opioid use produces an intense but rarely life-threatening withdrawal syndrome in both humans and experimental animals (Christie, 2008). Activation of opioid receptor will trigger several neuronal adaptations and several receptor-effector mechanisms which includes activation of receptor-operated potassium channels, inhibition of voltage-gated calcium channels and inhibition of adenylyl cyclase (Law *et al*., 2000; Koch and Hollt, 2008). Several in vitro and in vivo studies revealed that development of opioid tolerance in long-term exposure with morphine will lead to down regulation of receptors or counter regulatory processes such as adenylate cyclase superactivation (Varga *et al*., 2003; Zhao *et al*., 2006). It is also shown that cyclic Adenosine Monophosphate (cAMP) and its signalling pathways play a crucial role in the processing of painful stimuli. The mechanism involved in increasing and decreasing the cAMP level is generally associated with an increased nociception and have an analgesic effect (Finn and Whistler, 2001; Pierre *et al*., 2009). The cAMP superactivation was known as the cellular basis of opioid withdrawal (Berger and Whistler, 2010).

Thymoquinone (IUPAC name: 2-Isopropyl-5-methylbenzo-1,4-quinone) ; TQ is an aromatic ketone found in many medicinal plants. One of them is *Nigella Sativa* (NS) seeds. It is known to be an active phytochemical constituent in seeds of *Nigella sativa* or black cumin. Most properties of the whole NS seeds or their extracts are mainly attributed to quinone constituents of which thymoquinone is more abundant compound (Mahfouz *et al*., 1960; D'Antuono *et al*., 2002; Parvardeh and Fatehi, 2003). Recently, a great deal of attention has been given to this pharmacologically active quinone. TQ has been reported for its many therapeutic potential in a number of medical conditions (Ghayur *et al*.,

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2012). Currently, TQ had been shown to attenuates the development of morphine-induced dependence in mice by inhibiting brain oxidative stress and increase the expression of brain inducible Nitric Oxide (NO) synthase (Abdel-Zaher *et al*., 2013; Hosseinzadeh *et al*., 2016).

Thymoquinone also has potential chemical effect mimicking opioid, especially on the mechanisms of opioid dependency and tolerance. It's an opioid receptor stimulating compound with 45% ligand displacement at mu-opioid receptor which is receptor that is critical for morphine's rewarding effects.

Although, TQ were claimed to have the potential to reduce the opioid tolerance and dependence, there is no report on cellular mechanisms for chronic opioid treatment. In this study, the effects of TQ on the viability of opioid receptor expressing cells (U87 MG), the ability of TQ to reduce the upregulation of cAMP level in U87 MG cells after cotreatment with morphine for 48 h were investigated. We showed that TQ were not toxic to the cell lines.

MATERIALS AND METHODS

Opioid receptor expressing cells (U87 cells) were a gift from Animal Tissue Culture Lab, Faculty of Food Sciences and Bioresources, UniSZA. RPMI 1640 medium, Fetal Bovine Serum (FBS) and penicillin/streptomycin were obtained from Gibco (Invitrogen, USA). Dimethyl sulfoxides (DMSO) were purchased from Merck (Germany). Thymoquinone (>99% pure) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder was purchased from Sigma-Aldrich (USA). Morphine sulphate and methadone hydrochloride were purchased from Pharmaserv. The cAMP ELISA Kit were purchased from Cell Biolabs, Inc.

Dose response study (MTT assay): The dose response study of thymoquinone and morphine were evaluated using the colorimetric MTT assay. Briefly, 2×10^5 cells/mL were seeded on a 96-well plate in 100 μL culture medium per well. The cells were plated in triplicate. A serial dilution of TQ (6090, 3045, 1522.5, 761.25, 380.625, 190.3125 and 95.15625 μM) and morphine (3500, 1750, 875, 437.5, 218.75, 109.375 and 54.69 μM) were prepared in different concentrations All dilutions were transferred to the cells in the 96-well plate and incubated for 72 h. Subsequently, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg/mL) were added to the cells in the dark and incubated for 4 h, covered with aluminium foil. After incubation, DMSO (100 μL) was added to each well to dissolve the formazan crystals formed and absorbance was read at a wavelength of 570 nm as measurement wavelength and 630 nm as reference wavelength using the Tecan ELISA micro plate reader. The potency of cell growth inhibition for the test agents were expressed as the half maximal (50%) inhibitory concentration, IC_{50}. The amount of color produced is directly proportional to the number of viable cell. Cell viability rate was calculated as the percentage of MTT absorption as follows:

$$\text{Survival (\%)} = \left(\frac{\text{Mean experimental absorbance}}{\text{Mean control absorbance}}\right)\times 100$$

Cell culture and treatments: The opioid receptor expressing cells (U87 MG) was used to measure the effect of thymoquinone on the changes of intracellular cAMP concentration. U87 MG cells was grown in tissue culture flasks with RPMI 1640 medium containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (w/v) penicillin/streptomycin. U87 MG cells were grown as a mono-layer in a humidified incubator supplemented with 5% CO_2 at 37°C. Morphine and thymoquinone dose response studies were conducted by MTT assay. In order to demonstrate the morphine dependence and withdrawal, U87 MG cells were treated by removing the growth media and replacing with fresh growth media containing the following final concentrations of thymoquinone; 1218, 609, 305, 61 and 30 μM and morphine: 701, 350, 175, 35 and 18 μM. As a negative control, cells were treated with an equivalent volume of vehicle in growth media. The treatment groups were vehicle, morphine alone (35 μM), morphine and methadone (162 μM), morphine and TQ (61 μM) and morphine, methadone and TQ. Finally, the supernatants were harvested for measuring the intracellular cAMP concentration.

Measurement of intracellular cAMP concentration: Treated and non-treated U87 MG cells in 96 well plates were assayed for the intracellular cAMP concentration after incubation with morphine and thymoquinone using the kit. Briefly, under acetylated condition, the medium was removed and Lysis Buffer was added to the cells and incubated at 4°C for 20 min. The cells were scraped off from the plate and dissociated by pipetting up and down until suspension is homogeneous before centrifuged at top speed for 10 min. The supernatants were collected and assayed directly for cAMP by incubating the samples in the goat anti-rabbit antibody-coated plate. Diluted peroxidase cAMP tracer conjugate and rabbit anti-cAMP polyclonal antibody was then added to each tested well and incubated for 2 h with shaking at room temperature.

After washing for several times, substrate was added to the wells and incubate for 5-20 min on an orbital shaker at room temperature. The reactions were stopped by adding stop solution and the results were determined using a micro plate reader set to 450 nm as the primary wave length.

Statistical analysis: The cell viability was calculated using a computer software program developed by Microsoft Excell 2007 based on the optimal density readings at 570 nm. Results were presented as means ±SDs. Statistical analysis was done using one way Analysis of Variance (ANOVA) for grouped comparison followed by Dunnet's test and unpaired student's t-test for comparing unpaired samples. $p<0.05$ were considered statistically significant. The percentages of cell viability were presented graphically in the form of graph using a computer software program of Graph Pad Prism 7.

RESULTS AND DISCUSSION

***In vitro* cell proliferation assay:** The cytotoxicity of thymoquinone and morphine on U87 MG cells were determined using MTT assay. The dose response curve was plotted by plotting the percentage growth of cells vs. concentration of the compound. As shown in Fig. 1a, the IC_{50} value of thymoquinone was 87.1 μM. Thus, the thymoquinone concentrations used for subsequent experiments were 1218, 609, 305, 61 and 30 μM.

Morphine sulphate and thymoquinone induced regulation of cyclic AMP in U87 MG cells: U87 MG cells were treated with 701, 350, 175, 35 and 18 μM morphine or 1218, 609, 305, 61 and 30 μM thymoquinone for 3 and 48 h. Treatment of cells with morphine for 3 h downregulated the cAMP level. Result showed that concentrations at 350, 175, 35 and 18 μM of morphine gives the significant reduction in the cAMP level as compared to cell only (control) (Fig. 2 and 3). Meanwhile, treatment of cell with thymoquinone for 3 h showed 1218, 609 and 30 μM of thymoquinone gives the significant reduction in the cAMP level as compared to control (Fig. 4). By contrast, cells treated with morphine for 48 h produced chronic morphine addiction as is shown by the increased in the cAMP level as compared to control. These effects were observed when the cells were treated with morphine at lower dose levels, i.e., 35 μM (Fig. 3). Interestingly, cells pretreated with TQ for 48h did not showed significant increase in cAMP level as compared to 3h thymoquinone-treated cells in all concentrations tested (Fig. 5).

Effect of co-treatment of morphine sulphate and thymoquinone on cAMP regulation: In this study, the effect of thymoquinone on cAMP level and the interaction between thymoquinone and morphine were

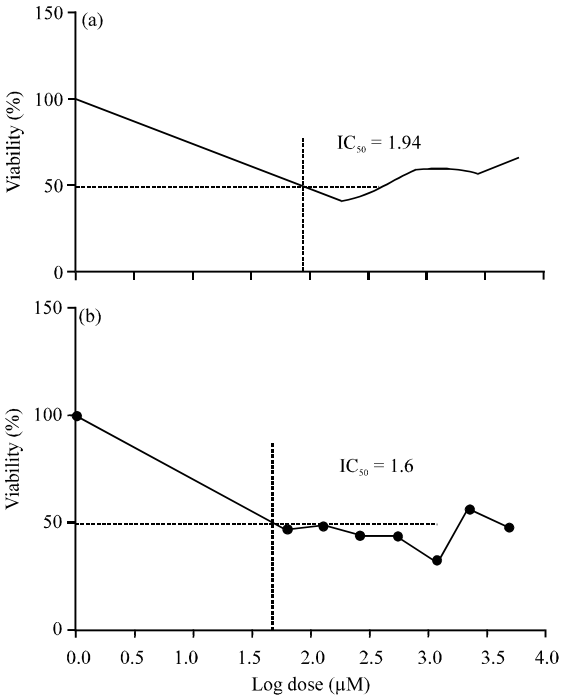

Fig. 1: The cytoxicity effect of thymoquinone and morphine on U87 MG cells. The cells were treated with a range of concentration 54.69-6090 μM and incubated for 72 h. The IC_{50} value of: a) Thymoquinone was 87.1 μM and b) Morphine was 39.81 μM. Data represent the mean±SD of three independent experiments

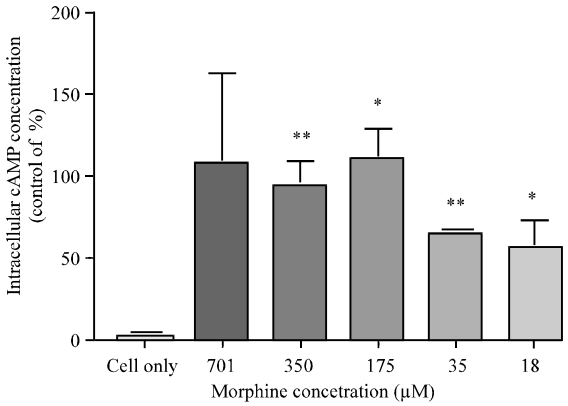

Fig. 2: Dose response effect of morphine on intracellular cAMP concentration after 3 h incubation. Three independent experiments were carried out and the data represent the mean ±SD. Statistical analysis was carried out using a student Dunnet's test. *p≤0.05 vs. control (cell only)

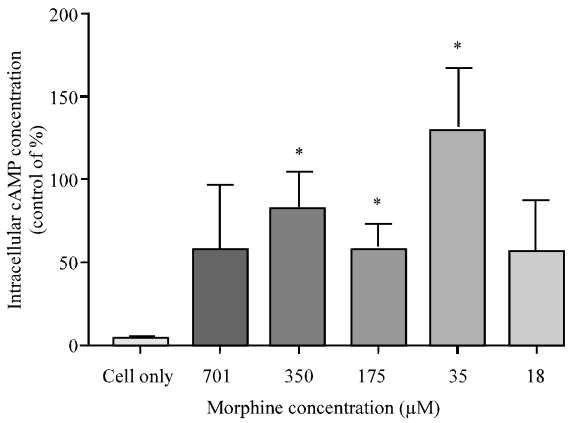

Fig. 3: Dose response effect of morphine on intracellular cAMP concentration after 48 h incubation. Three independent experiments were carried out and the data represent the mean ±SD. Statistical analysis was carried out using a student Dunnet's test.*p≤0.05 vs. control (cell only)

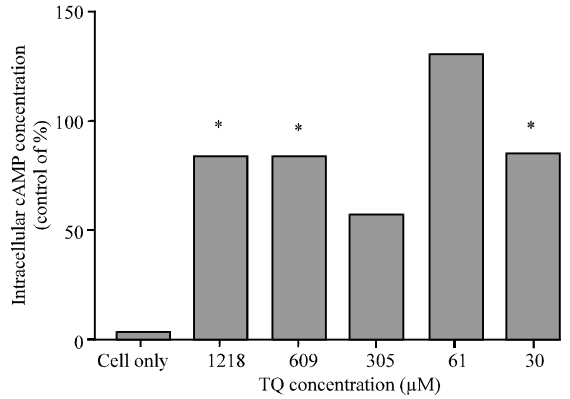

Fig. 4: Dose response effect of thymoquinone on intracellular cAMP concentration after 3 h incubation. Three independent experiments were carried out and the data represent the mean ±SD. Statistical analysis was carried out using a student Dunnet's test. *p≤0.05 vs control (cell only)

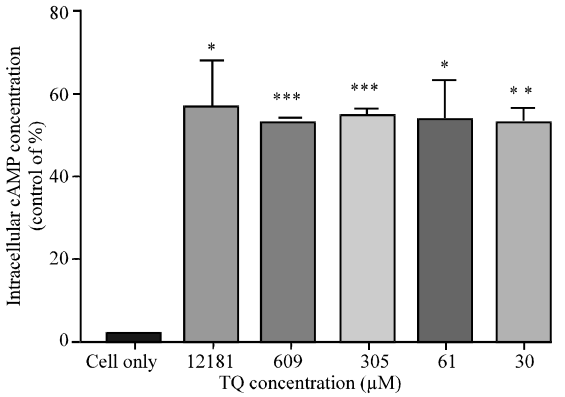

Fig. 5: Dose response effect of thymoquinone on intracellular cAMP concentration after 48 h incubation. Three independent experiments were carried out and the data represent the mean ± SD. Statistical analysis was carried out using a student Dunnet's test. *p≤0.05 vs. control (cell only)

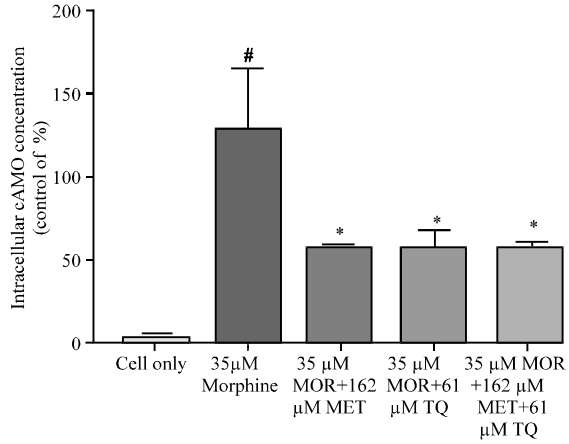

Fig. 6: Dose response effect of co-treatment of morphine sulphate and thymoquinone on intracellular cAMP concentration in U87 MG cells after 48 h incubation. Three independent experiments were carried out and the data represent the mean ± SD. (MOR = Morphine sulphate, MET = Methadone and TQ = Thymoquinone). Statistical analysis was carried out using a student Dunnet's test. #p≤0.05 vs control (cell only). *p≤0.05 vs. 35 μM morphine

investigated. U87 MG cells were treated with thymoquinone and morphine together for 48 h. To determine if thymoquinone will attenuate the increase in cAMP content produced by morphine, the U87 MG cells were cotreated with 35 μM of morphine and 61 μM of thymoquinone. The agonist opioids, Methadone was also used in this study as methadone is a drug used in treating opioid addiction. Methadone has the capability to block the compensatory increase in intracellular cAMP in chronic opioid treatment and naloxone-precipitated cAMP overshoot observed after chronic treatment with morphine (Liu *et al*., 1999). As expected, there was a clear reduction of cAMP content in morphine and methadone-treated U87 MG cells. As for morphine groups, withdrawal was induced by washing the morphine away and assayed for cAMP content. As a result, there was a surge in cAMP content when the drug was washed away. Thymoquinone

was also shown to inhibit the overshoot of cAMP content induced by morphine at concentrations 61 μM (Fig.6) as shown in morphine and thymoquinone-treated U87 MG cells. The result showed that thymoquinone has the potential to reduce the upregulation of cAMP level after chronic morphine treatment at lower concentration.

Nigella Sativa Linn (NS) belongs to family Ranunculaceae. The herb is widely known in different parts of the world and its seeds are used as condiment. In subcontinent it is known as 'kalonji' and its Arabic name is 'Habatul Sauda'. In the west it is known as "Black Cumin". There is a Hadith of Hazrat Muhammad (PBUH) that 'black seed is treatment of every disease but death'. In the present study, the potential of thymoquinone, a bioactive compound from NS as a drug substitution therapy for chronic morphine treatment on a regulation of MOR protein concentration in U87 MG cells was investigated. It is well understood that repeated exposure to drugs of abuse alters the amounts and even the types of genes expressed in specific brain regions (Nestler, 2004). In this aspect, altered expression of genes can mediate changes in the function of individual neurons and the larger neural circuits within which the neurons operate. Activation of any of the three opioid receptor subtypes produces common cellular actions. The most commonly reported actions include inhibition of adenylyl cyclase, activation of a potassium conductance, inhibition of calcium conductance and an inhibition of transmitter release (Williams *et al*., 2001). Other observations studied in the actions of opioids are the activation of Protein Kinase C (PKC), the release of calcium from extracellular stores, the activation of the Mitogen-Activated Protein Kinase (MAPK) cascade and the realization that receptor trafficking plays an important role in receptor function (Berger and Whistler, 2010).

In the present study, the effects of thymoquinone on the changes in cyclic cAMP in U87 MG cells were examined. We first identify the dose response effects of both morphine and thymoquinone on the changes in intracellular cAMP concentration and the supplement effects of thymoquinone on morphine-induced cAMP overshoot after chronic morphine treatment.

The opioid effect was attributed largely to the mu-receptor rather than the δ-receptor (Yu *et al*., 1999). Therefore, U87 MG cells are useful to study the efficacy and tolerance of narcotic analgesics. The short and the long term effects of morphine on intracellular cAMP concentration using different concentrations of morphine were examined. Our results showed that morphine at concentrations 35 μM gives significant inhibition of adenylyl cyclase activity and reduces cAMP level after short-term exposure (3 h) with morphine and eventually has contributed to the superactivation of adenylyl cyclase manifested by a rebound of cAMP concentration after long-term exposure (48 h) with morphine as compared to the normal and other different doses tested. Based on this study, we already demonstrate that withdrawal and dependence can be seen at cellular level following an acute and chronic treatment in U87 MG cells. Meanwhile, thymoquinone seems did not showed any significant rise of cAMP level compared to 3 h treatments of thymoquinone after incubating the cells for 48 h for any doses tested as shown in the results, suggesting that thymoquinone probably can reduce the effects of opioid dependence and withdrawal in this study.

A few studies had reported that thymoquinone has a potential to be used as a substitution therapy for chronic morphine treatment (Abdel-Zaher *et al*., 2010, 2013; Hosseinzadeh *et al*., 2016). Thus, we also studied the effect of co treatment of thymoquinone with morphine on intracellular cAMP concentration after 48 h treatment. The result of the present studies showed that co treatment of 61 μM thymoquinone with morphine significantly attenuate the increase in cAMP content produced by 35 μM morphine. The results of this study indicated that thymoquinone has a lower opioid effect and can prevent chronic morphine treatment-induced adaptive sensitization or overshoot of adenylate cyclase activity (Liu *et al*., 1999).

Co treatment of thymoquinone with Methadone also had been investigated in this study. The result showed that co treatment of thymoquinone with methadone also significantly attenuate the increase in cAMP content produced by 35 μM morphine in U87 MG cell. These results also suggested that methadone can prevent morphine-induced withdrawal and dependence (Liu *et al*., 1999). Methadone and morphine are similar opioid receptor agonists but methadone has a lower dependence potential than morphine and is effectively used in the treatment of opioid addiction whereas morphine induces dependence (Berger and Whistler, 2010). An up-regulation of the cAMP pathway is known to be the important adaptive changes induced by chronic exposure to opiate and is related to opiate dependence which have been demonstrated at the level of individual neurons chronically exposed to morphine such as SK-N-SH and SH- SY5Y cells which express abundant Mu-Opioid Receptor (MOR) (Yu *et al*., 2003; Mohan *et al*., 2010).

Previous report has shown that thymoquinone is an opioid receptor stimulating compound with 45% ligand displacement at mu-opioid receptor. Based on these ligands displacement, thymoquinone has the potential to be used as an agent for opioid addiction treatment. The present study has the limitation to understand the effect of thymoquinone on mu-opioid receptor expression level after chronic morphine treatment. Therefore, it will be interesting to study further the effect of thymoquinone on the desensitization of mu-opioid receptor and cAMP-Response Element Binding protein (CREB).

CONCLUSION

In summary, these findings suggest that thymoquinone could potentially attenuate the tolerance and dependence of chronic morphine treatment by suppressing the increase in cAMP content produced by 35 μM morphine. The findings also provide an understanding of the effect of thymoquinone on the cellular basis of substitution therapy for opioid addiction. It also provides an insight into the mechanisms of dependence and tolerance, thereby facilitating further studies on the design and development of highly effective and addictive substitute drugs for opioid addiction therapy.

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