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EFFECTS OF ZAMZAM WATER AND METHADONE ON THE EXPRESSION OF MU-OPIOID RECEPTOR-1 GENE IN MORPHINE-DEPENDENT RATS AFTER CHRONIC MORPHINE ADMINISTRATION

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Abstract

Background: Sodium ion is an essential ion that is implicated in many physiological functions. Recently, sodium ion was reported to facilitate the activation of Mu-Opioid Receptor (MOR) by binding at the allosteric site of the MOR. Zamzam water is water originated from Mecca. Couple of studies proved that Zamzam water has the therapeutic effect owing to its mineral. In this study, we want to determine the ion concentration of Zamzam water and then to investigate the effects of Zamzam water and co-treatment with methadone on the regulation of MOR-1gene after chronic morphine administration.
Materials and Methods: Zamzam water, tap water and normal mineral water were analyzed using Ion chromatography. Meanwhile, in animal study, 50 male Sprague Dawley rats were randomly divided into five groups. All group of rat were made dependence on morphine using intraperitoneal injection except for normal group. Morphine dependent rats then were treated with methadone, Zamzam water and co-treatment methadone with Zamzam water for thirty days, respectively. The Ventral Tegmental Area (VTA) of rat's brain was dissected and subjected to real-time quantitative RT-PCR to determine the regulation of MOR-1 gene expression. The obtained data were analyzed using SPSS v.11 software, and one-way ANOVA followed by Tukey's Post-test.
Results: The data obtained showed that Zamzam water is significantly high in ion concentration compared to tap water and normal mineral water. Besides, the result from gene expression analysis showed co-treatment Zamzam water and methadone significantly prevented the downregulation of MOR as compared to methadone and Zamzam water treatment alone ($P<0.05$). A possible explanation for this might be due to the presence of sodium ion in Zamzam water which activate MOR then promote the endocytosis of the MOR.
Conclusion: We concluded that co-treatment of methadone and Zamzam water significantly prevented downregulation of MOR-1 gene by promoting the endocytosis of MOR.

Keywords: Morphine, Zamzam water, methadone, morphine withdrawal symptoms, Mu-Opioid Receptor, Gene Expression

Introduction

Opiates such as morphine have been used as an analgesic for centuries. Morphine was recommended by the World Health Organization (WHO) in the management of cancer treatment (WHO, 1996). Unfortunately, its long-term use of morphine is very limited due to serious complication including unwanted side effects, tolerance, physical dependence and addiction. Acute and chronic administration of opiates drugs alters the gene expression in both brain and spinal cord.

It is believed that the alteration of certain gene expression caused by chronic exposure to opiates become the main factor that leads to the behavioral changes among the drug addicts (Zhu et al., 2012).

Methadone is a potent synthetic opiate agonist. It is widely being used for opioid substitution therapy because its effects are almost similar to morphine and other opiates (Abdel, 2006). A couple of studies reported that orally administered of adequate therapeutic doses of methadone could reduce morphine craving and block the onset of withdrawal (Breslin and Malone, 2006; Seymour et al., 2003). Tolerance and dependence are complex physiological responses that involve adaptations at multiple levels especially in the nervous system. Upon prolonged activation of Mu-Opioid Receptor (MOR); it leads to multiple cellular adaptations of downstream process. Previous studies reported that endocytosis of the MOR could prevent the morphine tolerance and dependence subsequently block the onset of withdrawal (Madhavan et al., 2010).

Zamzam water comes from well located in Makkah, the western province of the Kingdom of Saudi Arabia. Many studies have shown that Zamzam water is significantly high in mineral concentration, such as sodium, calcium as compared other normal mineral water (Alfadul and Khan, 2011; Khalid et al., 2014). Zamzam water was reported to have healing properties due to its high mineral contents. Our previous study shows that co-treatment Zamzam water and methadone significantly eliminated the spontaneous withdrawal symptoms like body weight loss, rare standing and sniffing (Halim et al., 2017). Hence, the aim of this study is to determine the ion concentration of Zamzam water and to investigate the effects of methadone, Zamzam water and co-treatment methadone and Zamzam water treatments towards the regulation of MOR-1 gene by using Real-Time PCR.

Materials and Methods

Sample collection

Zamzam water was obtained directly from Mecca, Saudi Arabia while tap water obtained from Chemistry Laboratory of University Sultan Zainal Abidin (UniSZA), Terengganu, Malaysia. Mineral water was purchased from the sundry shop in Kuala Terengganu, Malaysia.

Ion Chromatography Analysis

Ion analysis was carried out by Ion Chromatography (IC) (METROHM / 881 COMPACT IC PRO Model) and run for one hour to reach stable conditions. Standards of different concentrations were prepared to calibrate the IC using the calibration standards with certified METROHM for required parameters for Lithium (Li), Sodium (Na), Potassium (K), Calcium (Ca), Magnesium (Mg). Fluoride (F), Chloride (Cl), Nitrite (NO_{2-}), Nitrate (NO_{3-}), Bromide (Br), Phosphate (PO_{43-}) and Sulphate (SO_{4-2}). After calibration of the standards, samples were run and the concentration of the water samples were recorded as mg/l. The injection volume was 10 µl.

Animals

Fifty of Male Sprague-Dawley rats weighing 180–220 gram were utilized in the experiment. All animals were maintained under controlled conditions (23±1 °C, 12hr light/dark cycle, relative humidity of 30-40%). They were provided with food and water ad libitum, and subjected to a 2-week acclimatization period prior to the start of the studies. All animal studies were performed according to the guidelines endorsed by the Ethics Committee of Universiti Sultan Zainal Abidin (UniSZA) (BNU/EC/01/2011). At the end of the treatment duration, rats were sacrificed by decapitation and their brains were dissected rapidly for VTA regions. All samples were placed on dry ice and stored at -80°C until the days of assay.

Drug

Morphine sulphate (10mg/ml) and methadone hydrochloride (5mg/ml) were purchased from Merck (Germany). Morphine sulphate was dissolved in normal saline solution and was injected to the rats intraperitoneally. Meanwhile for treatment process, methadone syrup was dissolved in the distilled water and Zamzam water, respectively and then consumed by morphine dependent rat orally.

Induction of morphine dependence

In first day, rats were made dependent by repeated intraperitoneal injections of 10 mg/kg morphine at 08.00 AM and 19.00 PM. The morphine doses were increased daily by 2 mg/kg increments per day until a maximum of 68 mg/kg twice daily for 30 days was achieved (Ghowsi and Yousofvand, 2015).

Experimental groups

Rats were randomly divided into five experimental groups, each comprising 10 rats as follows; negative control group, received distilled water orally for 30 consecutive days. No morphine was injected into this group. In positive control group, rats were received variable doses of morphine sulphate solution for 30 consecutive days. Morphine was stopped after 30 days and no treatment was given in this group during the treatment period. Next, in the methadone treatment group, rats received variable doses of morphine sulphate solution for 30 consecutive days. Subsequently, the rats were given methadone orally as a treatment with increasing dose by 1 mg/100 mL per day until a maximum of 2.5 mg/100 mL for 30 days was achieved. In Zamzam water treatment group, the rats were received variable doses of morphine sulphate solution for 30 consecutive days. Then, the rats were supplemented with Zamzam water orally as a treatment for 30 consecutive days after day one of withdrawal. Last group, co-treatment methadone and Zamzam water group, rats were received variable doses of morphine sulphate solution for 30 consecutive days. Afterwards, the rats were orally given with methadone mixed with Zamzam water as a treatment with increasing dose by 1 mg/100 mL per day until a maximum of 2.5 mg/100 mL for 30 days was achieved.

Isolation of RNA

Tissue samples of rat's brain from the respective group were homogenized in 1 ml of TRIzol reagent. Total RNA was extracted with chloroform and isopropanol according to the manufacturer's instruction (ThermoFisher Scientific, USA). Then, the vacuum dried RNA was dissolved in 200 µl of DEPC-water and the quantity of total RNA was measured by the Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, USA). The integrity of total RNA and the absence of contaminating genomic DNA were confirmed by 1% agarose gel electrophoresis. The purified RNA with A260/A280 ratio of >-1.8 was subsequently used for cDNA synthesis.

cDNA synthesis

The cDNA synthesis was performed with an iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) using 1 µg of total RNA according to the manufacturer's instructions

Real-time quantitative RT-PCR

In this study, GADPH was used as a housekeeping gene and amplified in parallel with MOR-1 using real-time PCR. Log copy number was calculated from a fit standard curve using PCR baseline subtracted Ct value. The MOR-1 mRNA level in each sample was normalized as a ratio of MOR-1 mRNA/GAPDH mRNA. The changed folds of expressed MOR-1 mRNA level between control and treatment were expressed as a percentage of the control. Significance of the data was analyzed by one-way ANOVA and then compared by Tukey's Post-test multiple comparison tests using SPSS Ver.20. $P<0.05$ was considered to be statistically significant.

Results and Discussion

Table 1: Ion concentration in Zamzam water, tap water and normal mineral water samples

Sample/ concentration (mg/L)	Zamzam water	Tap water	Normal mineral water
Lithium	0.052	0.066	0.060
Sodium	94.432	1.970	8.609
Potassium	41.156	1.089	2.497
Calcium	65.545	2.667	38.469
Magnesium	12.113	0.786	3.387
Fluoride	0.41	0.29	0.65
Chloride	76.08	4.78	0.76
Nitrite	0.23	0.21	0.25
Bromide	0.77	0.36	0.367
Nitrate	36.23	0.75	0.253
Phosphate	0.62	0.60	ND
Sulphate	88.28	4.19	6.36

ND not detected

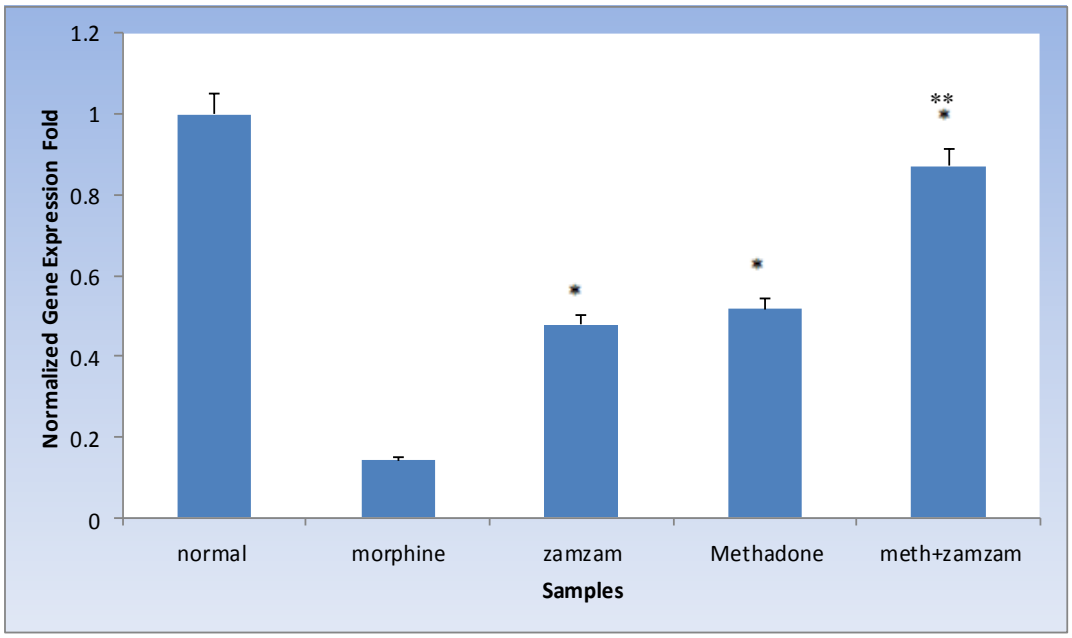

Figure 1: MOR-1 relative mRNA levels were quantified by real-time PCR using GAPDH as the reference gene. The results are the means ± SEM of a triplicate assay for three independent experiments. **Significance set at $p<0.05$.

Table 2: Sequence of the primers used in real time PCR for rat MOR-1 and GADPH.

mRNA accession (nucleotide position)	Primers	Fragment length (bp)
Oprm1	5'- ATCCAGTTCTTTACGCCTTCC -3'	126
Oprm1	5'- GATGTTCCCTAGTGTTCTGACG -3'	
GAPDH	5'- GTCTTCACCACCATGGAGAAGG -3'	197
GAPDH	5'- TCATGGATGACCTTGGCCAG-3'	

Table 3: Summary of MOR-1 mRNA levels by real-time PCR using GAPDH as the reference gene. The results are the means ± SEM of triplicate assay for three independent experiments. *Significance set at $p<0.05$

Group	**Mean + SEM**
Normal	1 ± 0.13
Morphine	0.1415± 2.33
Methadone	0.5176 ± 3.87
Zamzam water	0.4789 ± 2.76
Methadone + Zamzam water	0.8706 ± 2.62

In the table 1 above is a result of ion chromatography that were done. As can be seen from the data, the cation and anion; Ca, Mg, Na, K, Cl, Br, NO_{3-} and SO_{4-2} in Zamzam water is significantly higher compared to mineral water ($p<0.05$). However, none of this cation mineral of Zamzam water crossed the recommended maximum concentration limit guidelines of WHO standards and Dietary Reference Intakes (DRI).

This finding is in agreement with studies done by Shomar and Zuhair and Kounganian (Shomar, 2012; Al Zuhair and Kounganian, 2006) which showed ion mineral in Zamzam water naturally significant higher as compared to drinking or mineral water. A possible explanation for this result may be due to geography factor. Zamzam water well located in Saudi Arabia, which means a factor of dry climate cause water to evaporate by capillary action and lead to increase the concentration of ion in Zamzam water (Shomar, 2012).

Besides that, we performed the real-time quantitative RT-PCR technique to examine the changes in MOR-1 gene expression in VTA tissue regions of the rat's brain. It was observed that in untreated rats, the mRNA level of MOR-1 in VTA region decreased significantly ($P<0.05$) after chronic morphine administration for 30 days in comparison to the control group. This result is consistent with previous report which shown that chronic morphine administration on animal models cause down regulation of MOR-1. This down regulation contributed to the development of morphine dependence and tolerance (Abdel, 2006; Bhargava and Gulati, 1990; Tao et al., 1990, 1998; Ueda and Ueda, 2008). Moreover, morphine causes more elevated tolerance and physical dependence by observing the somatic signs of withdrawal as compared to enkephalin (DAMGO), methadone, or etorphine (Duttaroy and Yoburn, 1995; Walker et al., 2001; Grecksch et al., 2006; Kim et al., 2008).

Other than that, as expected, the current result also showed that methadone treatment group significantly prevented down regulation of MOR-1 when comparing to the untreated group ($p<0.05$). Although methadone and morphine are similar opioid receptor agonists but couples of studies indicate that methadone has lower dependence potential than morphine [17-20]. (Duttaroy and Yoburn, 1995; Walker et al., 2001; Grecksch et al., 2006; Kim et al., 2008). In addition, methadone has been effectively used in the treatment of opioid addiction (Berger and Whistler, 2010). Another study also found that sub analgesic doses of methadone mixed with morphine could significantly reduce the development of both tolerance and dependence (He et al., 2005).

In the past two decades, the researchers have sought to determine the protective mechanism to reduce or alleviate the development of tolerance and dependence. One of the possible mechanisms that have been proposed by several researchers is thru the regulation of opioid receptors by endocytosis process (He et al., 2002; Whistler et al., 1999). Like many other neurotransmitter receptors, MOR undergoes rapid endocytosis once activated by either endogenous or exogenous opioids. Endocytosis process started when the receptor is desensitized to the Mu-Opioid agonist and then the receptors are endocytosed into an intracellular compartment. Subsequently, they will be recycled to the cell surface followed by resensitizing the receptor to the agonist (Finn and Whistler, 2001; Whistler et al., 2002; Minnis et al., 2003; Yu et al., 2010).

However, unlike most MOP receptor agonists like methadone, morphine is unable to induce substantial MOP receptor endocytosis in both in vitro (Keith et al., 1996; Koch et al., 2001; Yu et al., 1997) and in vivo studies (Abbadie and Pasternak, 2001; Keith et al., 1998; Sternini et al., 1996; Trafton et al., 2000). The failure of morphine to promote endocytosis will cause a prolonged activation of MOR which trigger the homeostatic cellular and systemic adaptations such as overproduction of cAMP that manifested as tolerance and physical dependence (Berger and Whistler, 2001). Posa and colleagues proposed that removing MOR activated by morphine from cell surface will end this phenomenon (Posa et al., 2015). Unlike morphine, which fails to drive significant endocytosis of the MOR, methadone more closely mimics the endogenous opiates and promotes substantial endocytosis (Borgland et al., 2003; Milan-Lobo and Whistler, 2011). So, in this study, as shown in figure 1, methadone has proven to prevent the down-regulation of MOR as compared to untreated group and it is believed due to the ability of methadone to promote endocytosis, which restores the MOR in the VTA.

In the others hand, we also prove that in this study, the result showed that co treatment of Zamzam water with methadone significantly prevents down-regulation of MOR compared to methadone alone or Zamzam water alone. We postulated that Zamzam water has synergistic effects when given together with methadone. A possible explanation for these results might be that due to the presence of higher concentration of sodium in Zamzam water, which might influence the regulation of MOR. Sodium is a known endogenous allosteric modulator of MOR and recent studies revealed that both water molecules and sodium ions are crucial for the MOR's activation. Sodium and water molecule will bind towards the allosteric site of MOR subsequently activate MOR and probably promote endocytosis process (Katritch et al., 2014; Shang et al., 2014; Yuan et al., 2013).

The current study has several limitations that must be considered in the interpretation of the findings. A limitation in this study is due to the absence of the one more alkaline group which have similar concentration as Zamzam water. Hence, we can compare whether this alkaline water can give a significant result as Zamzam water or not. This limitation is taken into consideration for our upcoming studies. In conclusion, we have demonstrated that combination of methadone and Zamzam water reduce/prevent the development of morphine dependence. This is speculated to be mediated by up regulated of MOR after chronic morphine administration. We postulated that the higher mineral content in Zamzam water especially sodium plays a vital role in promoting and facilitating the activation and endocytosis process of MOR.

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Conflict of interest: The authors declare no conflict of interest pertaining to this study.

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