COMPARISON OF THE EFFECT OF CHRONIC TREATMENT OF MITRAGYNINE AND MORPHINE ON ANTINOCICEPTIVE BEHAVIOUR AND cAMP-PKA RIIβ GENES EXPRESSION IN MICE

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TABLE OF CONTENTS

CHAPTER 2:

OBJECTIVES OF THE STUDY

CHAPTER 3:

MANUSCRIPT

CHAPTER 4:

STUDY PROTOCOL

CHAPTER 5:

APPENDICES

LISTS OF TABLES

LISTS OF FIGURES

.

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ABSTRAK

Tajuk: Perbandingan kesan suntikan kronik di antara mitragynine dan morphine terhadap toleransi tahan sakit dan ekspresi gene cAMP-PKA RIIβ ke atas tikus.

Later Belakang: Mitragynine adalah salah satu agonist untuk opioid receptor seperti morphine. Kajian terdahulu membuktikan kesan pengambilan morphine secara kronik mengakibatkan toleransi tahan sakit dan ini dikaitkan dengan peningkatan ekspresi gene cAMP-PKA RIIβ. Oleh yang demikian, suntikan kronik mitragynine digunakan bagi membuat perbandingan dengan morphine, berdasarkan 1) toleransi tahan sakit terhadap stimulus hot plate dan 2) ekspresi gene cAMP-PKA RIIβ. Ini merupakan kajian pertama kali mengkaji ekspresi gene cAMP-PKA RIIβ untuk tikus yang dirawat menggunakan mitragynine.

Metodologi: Sejumlah 39 ekor tikus Swiss albino dibahagikan secara rawak kepada 3 kumpulan, di mana setiap daripadanya akan menerima suntikan dos berganda, di bawah kulit setiap hari selama 9 hari, sama ada suntikan mitragynine, morphine atau placebo. Kesan toleransi tahan sakit dinilai menggunakan dua kaedah 1) Kesan tahan sakit terhadap panas: Ini dinilai menggunakan hot plate pada suhu 52°C (52±2^{0}C), 30 minit selepas suntikan, sehingga tikus menunjukkan tanda-tanda kesakitan (melompat, menjilat kaki). Masa tindak balas terhadap stimulus dikira sebagai peratusan tindak balas maksimum (MPE%) dan penurunan lebih 50% daripada purata %MPE dikira sebagai toleransi (penurunan) tahan sakit. 2) Kajian molekular: Tikus dikorbankan untuk

pengambilan sampel tisu otak (thalamus, medulla, PAG) bagi kajian ekspresi gene cAMP-PKA RIIβ menggunakan kaedah semi-quantitative PCR.

Keputusan : Tikus yang dirawat mitragynine tidak menunjukkan toleransi tahan sakit selepas 9 hari berbanding placebo ($p<0.01$), sama seperti morphine. Tetapi, ekspresi gene cAMP-PKA RIIβ untuk tikus dirawat morphine adalah lebih tinggi berbanding mitragynine dan placebo.

Kesimpulan: Suntikan kronik mitragynine tidak menunjukkan tanda toleransi tahan sakit terhadap stimulus hot plate dan ekspresi gene cAMP-PKA RIIβ berbanding morphine.

ABSTRACT

Title: Comparison of the Effect of Chronic Treatment of Mitragynine and Morphine on Antinociceptive Behaviour and cAMP-PKA RIIβ Genes Expression in Mice

Background: Mitragynine is an opioid agonist similar to morphine. Chronic administration of morphine is shown to increase the expression of cAMP-PKA RIIβ responsible for the development of tolerance. There were no previous studies done on the effect of chronic mitragynine administration on cAMP-PKA RIIβ gene expression. Therefore, this study aims to evaluate the development of tolerance in mice chronically treated with morphine and mitragynine, assessed by 1) antinociceptive behavior and 2) cAMP-PKA RIIβ gene expression.

Methods**:** A total of 39 Swiss albino mice were randomized into 3 groups, each of which received daily subcutaneous injection of either mitragynine, morphine or placebo, in escalating dose for 9 days. The development of tolerance was assessed by 1) Antinociceptive behaviour study: Antinociceptive response to thermal noxious stimuli was evaluated daily using hot plate test at 52^0C (52 ± 2^0C), 30 minutes after drug administration, until the signs of pain (hind-paw licking, jumping) exhibited. The antinociceptive response was quantified as a percentage of maximal possible effect (%MPE) and reduction in mean %MPE more than 50% was used for assessment of antinociceptive tolerance. 2) Molecular study: The cerebral tissues (thalamus, medulla, PAG) were dissected for sampling in order to determine cAMP-PKA RIIβ gene expression using semi-quantitative PCR method.

***Results*:** Mitragynine-treated mice did not show tolerance by day 9 when compared to placebo ($p<0.01$), similar to morphine. However, at molecular level, the cAMP-PKA RIIβ gene expression for morphine was enhanced compared to mitragynine and placebo.

***Conclusion*:** Chronic treatment with mitragynine did not show development of tolerance on antinociceptive behavior and cAMP-PKA RIIβ gene expression, same as placebo, whereas morphine showed an increased in expression of cAMP-PKA RIIβ gene despite no behavioural antinociceptive tolerance.

LIST OF ABBREVIATION

AC	Adenylyl cyclase
cAMP	Cyclic-adenosine monophosphate
cDNA	Complement deoxyribonucleic acid
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DOP	Delta opioid receptor
KOP	Kappa opioid receptor
MOP	Mu opioid receptor
MPE	Maximal possible effect
PCR	Polymerase chain reaction
PKA	Protein kinase A
PAG	Periaqueductal gray
RNA	Ribonucleic acid

CHAPTER 1

INTRODUCTION

1.1.Introduction

Opioid is a gold standard analgesia in medicine used in the treatment of acute and chronic pain. It is an exogenous substance which has an affinity for opioid receptors. When opioid binds to the opioid receptor, it inhibits neurotransmission by presynaptic reduction in excitatory neurotransmitter release leading to analgesia and other opioid-related effects (1). One of the theory postulated for the reduction in neurotransmitter release is as a result of inhibition of adenylyl cyclase (AC) leading to reduced intracellular cyclic Adenosine Monophosphate (cAMP) formation. However, this mechanism occurs only when opioids are given in acute administration. In chronic opioid use, this cAMP pathway instead is enhanced via the superactivation of AC (2) and thus is responsible for the development of tolerance to the opioid analgesic effects.

The opioid receptors can be found in central nervous (CNS) as well as outside the CNS in the peripheral tissues (1). They are classified as *mu* (MOP), *kappa* (KOP) and delta (DOP)(1). MOP is the main opioid receptor located throughout the CNS (cerebral cortex, basal ganglia, spinal cord, periaqueductal gray (PAG)). Its stimulation produces analgesia, respiratory depression, constipation and cardiovascular depression. Morphine is an opioid agonist which exerts its action primarily on MOP receptors and thus responsible for most of the opioid unwanted side effects (1).

Mitragynine is a *major* active compound found in *Mitragyna speciosa* Korth leaves (also known as ketum).It has been a great interest worldwide particularly among Asian scientists when it was discovered to demonstrate an agonist activity on opioid receptors (3)(4). It is however, structurally different from morphine. The plants thrive most abundantly in Southeast Asia

including Malaysia and traditionally, the plant has been consumed especially among farmers and laborers in Thailand to increase endurance and overcoming burden of their hard work (5). Moreover, it is often used to manage opium addiction (6).

The discovery of *M. speciosa* Korth extract and its alkaloids has propelled further researches to look into their action on different opioid receptors and behavioral changes. The focus of these researches was on its antinociceptive effect in acute doses. Previous study showed an equipotent dosage of *M. speciosa* Korth extract 200 mg/kg to morphine 5 mg/kg given orally (7). Other comparison studies on mitragynine and morphine dosage given intracerebroventricular in acute doses showed morphine has 3 times potency (8).

There was one study which showed the analgesic effect of chronic treatment of morphine was enhanced when combined with mitragynine via intraperitoneal injection and the effect of the combination showed less development of tolerance (9). However, to this date, there are no data published on equipotent dose of mitragynine to morphine for chronic administration via subcutaneous. It is therefore the aim of this study to evaluate the effect of chronic administration of mitragynine via subcutaneous route and the corresponding dose to morphine as well its effect on the development of antinociceptive tolerance.

1.2 Problem statement

Accurate assessment of pain remains a challenge as many measurement tools are often rely on psychophysical measurement that are not clearly defined in terms of ongoing pain versus stimulus evoked pain. Nor are they exclusive. Moreover, pain threshold differs among patients. In other word, pain is a subjective description based on certain parameters that largely based on individual experiences. Therefore, it is not surprise that the use of animal pain model has become more important in pain studies.

In clinical practice, the use of morphine has long been the important treatment of chronic pain like cancer pain. Unlike in acute pain treatment, where the emergence of other class of drugs gives physicians better options to tailor to patients' need and condition, the use of morphine in chronic pain is inevitable. This poses a clinical dilemma because chronic treatment with morphine has many undesirable side effects such as nausea and vomiting, constipation, respiratory depression and physical dependence.

In this light, the discovery of an alternative analgesic to morphine has generated many research interests worldwide, particularly among Asian scientists where the plants are indigenous. However, there is a lack in the study regarding the development of tolerance to antinociception in chronic treatment of mitragynine. The comparison study between chronic administration of mitragynine and morphine and its effect on development of antinociceptive tolerance should serve as a catalyst for further understanding of the pharmacodynamic of the mitragynine.

1.3 Justification of the study

Tolerance occurs with repeated administration of opioid drugs. It can be defined as a state of adaptation in which prolonged exposure to a certain drugs leads to decreased biological efficacy of drug action (10). As a result, opioid dosage needs to be increased in order to maintain its clinical efficacy. Increased dosage also means increased undesirable and hazardous side effects including addiction. Several theories on the mechanism of opioid tolerance development have been proposed based on cellular and molecular level researches. Of these theories, the superactivation of cAMP – dependant protein kinase A (PKA) signaling cascade has long been recognized as a typical molecular adaptation to chronic opioid administration (2).

cAMP- PKA is a holoenzyme which contains regulatory (R) and catalytic (C) subunits which are further differentiated into four regulatory (RIα, RIIα, RIβ, RIIβ) and four catalytic (Cα, Cβ, Cγ, PrKX) subunits respectively (11). The most recent study had identified RIIβ subunit to be predominantly expressed in a subgroup of sensory neurons including most neurons expressing nociceptive markers (12). Therefore, increased in cAMP levels will similarly raise the cAMP-PKA RIIβ subunits. Hence, in this regard, cAMP-PKA RIIβ gene expression was used to evaluate the development of tolerance to antinociceptive effect of morphine as well as mitragynine in chronic drug administration.

Because this study interest is in evaluating the relationship between behavioral changes and molecular adaptation to the chronic opioid treatment of both mitragynine and morphine, brain tissues sampling for RNA extraction were isolated from cerebral tissue (PAG, Thalamus, Medulla) and cAMP-PKA gene expression was quantified by using primers for cAMP-PKA RIIβ subtype.

To quantify the cAMP-PKA gene expression, this study employed semi-quantitative PCR method. For the reference gene expression, β-actin, a housekeeping gene, was used.

CHAPTER 2

OBJECTIVES

GENERAL OBJECTIVE

To compare the effect of chronic treatment of mitragynine and morphine on anti-nociceptive behaviour and cAMP-PKA RIIβ genes expression in mice.

2.2 SPECIFIC OBJECTIVES

2.2.1 To determine individually the onset of antinociceptive tolerance in mice treated with mitragynine and morphine

2.2.2 To compare the onset of antinociceptive tolerance in mitragynine-treated group and morphine-treated group

2.2.3 To compare the effects of chronic treatment of mitragynine and morphine on cAMP-PKA RIIβ gene expression in mice

2.3 STUDY HYPOTHESIS

2.3.1 H0: There is no difference in onset of tolerance to noxious stimuli in mitragynine and morphine-treated mice.

2.3.2 H0: There is no difference in cAMP-PKA RIIβ genes expression in mitragynine and morphine-treated mice.

CHAPTER 3
MANUSCRIPT

Title

Article Title: Comparison of the Effect of Chronic Treatment of Mitragynine and Morphine on Antinociceptive Behaviour and cAMP-PKA RIIβ Genes Expression in Mice

Running Head:

Chronic mitragynine treatment does not increase cAMP-PKA RIIβ genes expression in mice

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Abstract

Title: Comparison of the Effect of Chronic Treatment of Mitragynine and Morphine on cAMP-PKA RIIβ Genes Expression in Mice

Background: Mitragynine is an opioid agonist similar to morphine. Chronic administration of morphine is shown to increase the expression of cAMP-PKA RIIβ responsible for the development of tolerance. Therefore, the aim of this study was to investigate the effect of chronic administration of mitragynine on the expression of cAMP-PKA RIIβ genes in mice.

***Methods*:** A total of 39 Swiss albino mice were randomized into 3 groups, each of which received daily subcutaneous injection of either mitragynine, morphine or placebo, in escalating dose, for 9 days. Antinociceptive response to thermal noxious stimuli was evaluated daily using hot plate test (BIOSEB Cold-Hot Plate Analgesimeter), 30 minutes after drug administration, until pain-related behaviors exhibited. The antinociceptive response was quantified as a percentage of maximal possible effect (%MPE) and reduction in mean %MPE more than 50% was used for assessment of antinociceptive tolerance. The cAMP-PKA RIIβ gene expression was then quantified using semi-quantitative PCR (Phusion High-Fidelity DNA Polymerase Kit, by Thermo Fisher Scientific, USA) to further evaluate the antinociceptive tolerance development. β-actin was used as a housekeeping gene for cAMP-PKA.

***Results*:** Overall, the expression of cAMP-PKA RIIβ gene showed similar pattern between mitragynine-treated mice and placebo, but an increased in gene expression was shown in morphine-treated mice. For morphine, the gene expression in medulla was higher than in PAG.

***Conclusion*:** Chronic administration of opioid agonist, mitragynine was not associated with an increased in cAMP-PKA RIIβ genes expression in mice. The genes were expressed prominently in PAG compared to medulla oblongata.

***Keywords**: mitragynine, morphine, antinociceptive tolerance, cAMP-PKA RIIβ gene expression, opioid receptor*

Introduction

Mitragyna speciosa Korth also known as ketum, is a plant thrives most abundantly in Southeast Asia including Malaysia. Traditionally, the plant is consumed especially among farmers and laborers in Thailand to increase endurance and overcoming the burden of their hard work (1). Moreover, it is often used to manage opium addiction (2). *Mitragyna speciosa* Korth has been a great interest worldwide particularly among Asian scientists when its major alkaloid, mitragynine was discovered to demonstrate an agonist activity on opioid receptors(3) (4) . It is however, structurally different from morphine.

The discovery has propelled further researches to look into the action of *M. speciosa* Korth extract and its alkaloids on different opioid receptors and their behavioral changes. The focus of these researches was on its antinociceptive effect in acute doses. Previous study showed an equipotent dosage of M. speciosa Korth extract 200 mg/kg to morphine 5 mg/kg given orally (7). Other comparison studies on mitragynine and morphine dosage given intracerebroventricular in acute doses showed morphine has 3 times potency (8). However, to this date, there are no data published on the effect of chronic mitragynine administration via subcutaneous. It is therefore the aim of this study to evaluate the effect of chronic administration of mitragynine via subcutaneous route.

Moreover, this study interest is to evaluate the relationship between behavioral changes and molecular adaptation to the chronic opioid treatment of both *mitragynine* and morphine. Therefore, brain tissues sampling for RNA extraction were isolated from cerebral tissue (Periaqueductal Gray PAG, Thalamus, and Medulla). The expression of cAMP-PKA RIIβ gene, which is thought to be predominant isoform in many neurons (5), and postulated to be involved in the opioid tolerance, was then quantified by using primers for cAMP-PKA RIIβ

subtype through semi-quantitative PCR method. For the reference gene expression, β-actin, a housekeeping gene, was used.

Mitragynine as an opioid receptor agonist

Many studies have been conducted on mitragynine as well as other *M. speciosa* alkaloids showing opioid-like activities of these compounds (6)(7). Other studies revealed that mitragynine antinociceptive effect was inhibited by naloxone through supraspinal opioid receptors (3) and other opioid antagonists (8), implicating the opioid receptor system plays a major role as a primary mediator. Recent finding found that mitragynine acts as a partial agonist at the human MOP and competitive antagonists at the KOP and DOP (9). Because of the unique molecular pharmacology of this opioid agonist, further data need to be made available to look into its long term effects on the development of opioid tolerance.

Development of tolerance after chronic administration of opioid receptor agonists

Tolerance occurs with repeated administration of opioid drugs. As a result, opioid dosage needs to be increased in order to maintain its clinical efficacy. Increased dosage also means increased undesirable and hazardous side effects including addiction. Several theories on the mechanism of opioid tolerance development have been proposed based on cellular and molecular level researches (opioid receptor modulation, intracellular signal transduction and gene expression). The long-lived typical molecular adaptations that have been extensively studied is the superactivation of cyclic Adenosine Monophosphate (cAMP) - dependant protein kinase A signaling cascade (10).

Binding of opioid agonist to the opioid receptor inhibits neurotransmission by presynaptic reduction in excitatory neurotransmitter release leading to analgesia and other opioid-related effects (1). One of the theory postulated for the reduction in neurotransmitter release is as a result of inhibition of adenylyl cyclase (AC) leading to reduced intracellular cyclic Adenosine Monophosphate (cAMP) formation.

In chronic opioid use, this cAMP pathway instead is enhanced via the superactivation of AC (2) and thus is responsible for the development of tolerance. Until now, there are numerous data available to explain the various molecular mechanisms that underlie the chronic use of classical opioid like morphine. However, data regarding the effect of chronic mitragynine administration on tolerance is still lacking and warrants further investigations.

Methodology

Experimental Animals

Adult Swiss Albino mice, all male, aged 4-5 weeks old, weighted 20-40 grams were used in this study. All mice were sourced from Laboratory for Animal Research Unit (LARU) USM Health campus. Mice were drug and tested naïve prior to the study. 39 mice were chosen randomly and housed at 6 and 7 mice per cage (2 cages per study group), in a controlled temperature with lights on from 0730 to 1930 (12 hour light/dark cycle) and free access to food and water for at least 1 week before experiment. All mice were habituated to the testing apparatus several hours on the testing day. All experiments performed had been conducted in compliance with the LARU and Animal Ethic Committee (AEC) of USM guidelines.

Experimental materials and drugs

Mitragyna speciosa leaves were harvested and processed into methanol extract mitragynine powder by Prof. Dr. Sharif Mahsufi Mansor from The Center For Drug Research (CDR), Universiti Sains Malaysia, Pulau Pinang. The dried extract were sealed in a bottle and stored in the refrigerator at 4°C until it was used. The dried mitragynine extract was insoluble in saline solution; hence it was suspended in 20% Tween 20 and stored at 4°C until used.

The mitragynine solution was prepared in concentration of 90mg/kg body weight in escalating dosage; day1 90 mg/kg, day2 90mg/kg, day3 90mg/kg, day4 180mg/kg, day5 180mg/kg, day6 180 mg/kg, day7 360mg/kg, day8 360mg/kg and day9 360mg/kg (modified from Bryan et al, 2006). Morphine hydrochloride was suspended in 20% Tween 20 and concentration of 10mg/kg body weight was used and increased in escalating dose daily. (Day1 10mg/kg, day 2 10mg/kg, day 3 10mg/kg, day 4 20mg/kg, day 5 20mg/kg, day 6 20mg/kg, day 7 30mg/kg, day 8 30mg/kg, day 9 30mg/kg). Plain 20% Tween 20 was used as a vehicle.

All drugs were administered subcutaneously. Solution was injected in a volume of 10ml/kg body weight.

Experimental procedures

Assessment of pain

First introduced by Eddy and Leimbach in 1953. The. Pain assay was measured daily 30 minutes post drug administration, using hot plate (BIOSEB Cold-Hot Plate Analgesimeter) at 52°C (52±2°C) .Mice were placed on the hot plate surface and the latency period was recorded when pain related behaviors (paw licking, jumping, tail flick) occurred, by a stopwatch to the nearest 0.1 second. Pre-drug injection nociceptive threshold was measured three times and mean of reaction time was used as pre-drug latency. Prior to treatment, only mice that showed response within 18 seconds were selected for this study. Cut off latency of 45 seconds was employed as end point of analgesia to prevent tissue damage.

Antinociception was quantified as the percentage of maximal possible effect (MPE):

$$\text{MPE (\%)} = \frac{\text{Post-Drug Latency} - \text{Pre-Drug Latency}}{\text{Cut off time} - \text{Pre-Drug Latency}} \times 100$$

Euthanasia of the animal

At the end of tolerance assessment, all mice were euthanized by decapitation at the base of the skull under deep pentobarbital anesthesia. Pentobarbital 200 mg/kg body weight was administered intraperitoneally to render mice unconscious prior to decapitation.

Tissue Sampling and Storage

Post decapitation, brain tissues were immediately removed for dissection of cerebral tissue (PAG, Thalamus, and Medulla) and placed in RNAlater preservative. All fully frozen tissues were placed in labelled cryotubes and stored at -80°C until RNA extraction was performed.

Total RNA extraction and RNA quantification

Total RNA was isolated from cerebral tissue with Trizol Invitrogen kit according to the manufacturer's specifications.

RNA recovery samples were measured by spectrophotometry. The concentration of total RNA was determined with NanoDrop by measuring absorbance at a wavelength of 260 nm (A260) and it's purity was assessed by the ratio of the absorbance values at 260 and 280 nm, wherein a ratio of about 2.0 was considered a good indicator of purity. Upon completion of the manufacturer's protocol, RNA was aliquot then stored at (-80°C) until used.

cDNA Synthesis (According to High Capacity cDNA Reverse Transcription Kit)

cDNA synthesis mix was prepared according to manufacturer's specifications as follows :

Component	1 Reaction
Nuclease free water	4.2 µL
10X RT buffer	2 µL
10x Random Primers	2 µL
Reverse Transcriptase	1 µL
25x dNTP Mix	0.8 µL
Total	10 µL

10 µL RNA sample was added to 10 µL cDNA synthesis mix in each reaction tube (total volume of 20 µL in each tube) and was centrifuged to eliminate any air bubbles. Termination of reaction was carried out at 85°C for 5 min. The synthesized cDNA was then stored in -20°C or used for PCR immediately.

Semi-quantitative PCR

Semi-quantitative PCR was performed to measure gene expression of cAMP- PKA. β-actin was used as the reference gene for cAMP-PKA. Each PCR was performed in a total volume of 20 µL.

For cAMP, PCR method used to measure the gene expression is according to Phusion High-Fidelity DNA Polymerase Kit and the components are as follows:

Component	20 µL Reaction
5X Phusion HF Buffer (containing 7.5 mM MgCl2)	4 µL
10 mM dNTPs	0.4 µL
Forward primer	1.0 µL
Reverse primer	1.0 µL
Template DNA (cDNA)	1.0 µL
Phusion DNA Polymerase	0.2 µL
Deionize water	12.4 µL

Forward and Reverse cAMP-PKA RIIβ primers:

Sense primer: 5’-GTTTGTGGAGATGCCAAGCAG-3’

Anti-Sense primer: 5’-GCCACTCGATTACACAGGCT-3’

Product length: 570 bp

Initial denaturation step of 30 seconds at 98°C was followed by 30 cycles of: 15 seconds 98°C, 30 seconds 60°C, 1 minute 72°C and end step 2 minutes at 72°C.

For Beta Actin, the PCR method used is according to PCR SuperMix Kit:

Component	20 µL Reaction
PCR Supermix	17 µL
Forward primer	1.0 µL
Reverse primer	1.0 µL
Template DNA (cDNA)	1.0 µL

Forward and Reverse β-actin primers:

Sense Primer located in exon 6: 5’-GGCCAGGTCATCACTATTG -3’

Anti-sense primer in exon 7: 5′- GAGGTCTTTACGGATGTCAAC-3’

Product length: 147 bp

Initial denaturation step of 2 minutes at 94°C was followed by 30 cycles of: 15 seconds 94°C, 30 seconds 58°C and end step 1 minute 72°C.

Gel Electrophoresis

The PCR products was subjected to pre-stained 2.5% agarose gels in TBE buffer. This procedure is an established PCR procedure done in USM School of Medical Sciences.

Statistical analysis

All numerical data were expressed as mean and 95%CI. Statistical analyses were performed using SPSS version 22. Repeated measures ANOVA was used to compare the differences between the means of the antinociceptive response (%MPE) within all groups. One-way ANOVA followed by Bonferroni multiple comparison test were used to compare means of more than 2 groups. P value < 0.05 is considered statistically significant. The protein bands for cAMP-PKA RIIβ genes were analysed using Image J software.

3.5 Results

There was no difference in the size or weight of all mice selected in the study ($p > 0.05$). One mouse from mitragynine group was excluded from the study due to right hind-paw skin infection. Two mice died on day 1 and day 8 after treated with mitragynine at a dose of 90mg/kg and 360mg/kg respectively. Both mice were also excluded from the study.

3.5.1 Antinociceptive tolerance

There was significant difference in the antinociceptive response, %MPE, between all the groups, Fstat= 83.77 (2, 33), p-value <0.001. Mice receiving chronic treatment of mitragynine and morphine showed significant difference in %MPE when compared to placebo ($p< 0.001$). Both mitragynine and morphine-treated group did not demonstrate the onset of antinociceptive tolerance to hot plate test after 9 days of chronic drug administration.

3.5.2 Expression of cAMP-PKA RIIβ gene

Mitragynine-treated group did not show expression of cAMP-PKA RIIβ gene at medulla oblongata similar to placebo, and less expression at PAG when compared to morphine. Whereas morphine-treated group showed increased in gene expression at medulla more than PAG.

3.6 Discussion

Prolonged exposure to opioid drugs reduce the analgesic effects of this drugs leading to development of tolerance. The present study evaluate the development of tolerance based on >50% reduction in antinociceptive response or MPE, as well as expression of cAMP-PKA RIIβ gene after 9 days of chronic drug administration. This is the first study looking on the relationship between antinociceptive behaviour and molecular changes in chronic mitragynine administration.

Effect of chronic administration of mitragynine on antinociceptive behavior and cAMP-PKA RIIβ gene expression in mice

The present results have shown that mitragynine did not demonstrate a reduction in antinociceptive response even at the high drug dose of 360mg/kg. Instead, the response was increased with escalating dosage of mitragynine. These findings suggested that there was no onset of tolerance developed based on the antinociceptive behaviours observed after 9 days of drug injection.

In the previous study (11) tolerance to mitragynine antinociceptive effect was assessed using cAMP and cAMP response element binding (CREB) protein expression by immunoassay kit. CREB is a transcription factor that will be phosphorylated in response to cAMP, promoting cellular genes expression. The study found that mitragynine alone did not increase the level of expression of both proteins indicating that there was no development of tolerance, and the expression was less in combination with morphine. However, it was performed at a lower drug dose (15 and 25mg/kg) via intraperitoneal injection and without escalation of the dose for 9 days.

This present study utilized semiquantitative PCR method to quantify cAMP-PKA RIIβ gene expression. The up-regulation of the cAMP-PKA RIIβ gene was postulated to be associated with opioid tolerance. In the present findings, mitragynine group showed a similar pattern of gene expression to placebo (band density of 1.02) at medulla oblongata. Even so, the gene was expressed in about the same density for both mitragynine (1.20) and morphine (1.22) at PAG region. Overall, the development of tolerance to mitragynine was not evident at medulla.

There are few postulations that may explain the reason for the variation in the gene expression pattern at the medulla, PAG and thalamus in this study. Even though the cAMP-PKA RIIβ is predominantly expressed in nociceptive neurons, it is expressed at different levels in most areas of the brain (12). Apart from that, mitragynine may also be thought to take different pathways distinct from other opioid agonists (via non-opioid receptors like alpha-2 adrenergic, adenosine A2, and dopamine D2 receptors), in which this in some degree, could contribute to the disparity of the results, either for its antinociceptive effect or tolerance development. With respect to the duration used for the chronic treatment of mitragynine, there may be a possibility of inadequate time studied and a longer time needed to see the effect in gene expression at molecular or cellular level. The same or reduced dose threshold without escalation of the dose of mitragynine (modified from Bryant et al) could be adjusted and used so as to elicit tolerance and more expression of the gene. Lastly, it can be concluded that the band density was not seen possibly due to mitragynine true intolerance itself.

Development of tolerance for morphine

Meanwhile, morphine treated mice in this study also showed no development of tolerance based on the antinociceptive behaviors. Rather the antinociceptive response was at

the maximal throughout the 9 day treatment. Nevertheless, the overall relative band density for cAMP-PKA RIIβ gene was higher compared to mitragynine and placebo group at both medulla oblongata and PAG, suggesting greater tolerance development in this treatment group.

To this date, there was no conclusive term to define chronic opioid treatment in the animal study. Some previous studies showed antinociceptive tolerance to morphine developed by day 9, with the twice daily administration (13)(11). While other study showed tolerance to morphine by day 10 (14). Moreover, morphine analgesic tolerance was found to be heritable, with some mice strain showed robust tolerance while others showed very little tolerance (15). As for this study, the results for antinociceptive tolerance may still be subjected to intra-strain variability which could affect the variance equality in the sample, despite the use of standardized methods in habituation and handling of the animals. The methods of tolerance assessment also have an impact on tolerance detection (16). The PCR methods utilized in this study have not yet been well studied before. Thus, further researches to compare the reliability and effectiveness of various methods are warranted.

In conclusion, the antinociceptive behavior in mitragynine-treated and morphine-treated mice does not show signs of tolerance. On molecular level, however, morphine-treated mice showed an increased level of cAMP-PKA RIIβ gene by one fold despite no tolerance on antinociceptive behavior. Whereas mitragynine showed similar expression of cAMP-PKA RIIβ gene to placebo.

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