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In vitro effect of mitragynine on activity of drug metabolizing enzymes, n-demethylase and glutathione s-transferase in streptozotocin-induced diabetic rats

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Abstract

Mitragynine is the major alkaloid of *Mitragyna speciosa* Korth. It is responsible for antinociceptive, antidepressant like effects and used as substitute for morphine to treat opium withdrawal. *Mitragyna speciosa* plant belongs to Rubiaceae family. In Malaysia, the leaves of *Mitragyna speciosa* are used to treat diarrhea, pain and as cough suppressant. Following experiments were undertaken to evaluate the effect of mitragynine on aminopyrine N-demethylase and glutathione S-transferase activity in diabetic Sprague-Dawley (SD) male and female rats. Different concentrations of mitragynine (0.0025µM-250µM) were used to evaluate activity of both enzymes. Collagenase perfusion technique was used to isolate hepatocytes and aminopyrine N-demethylase activity was determined in hepatocytes by measuring the quantity of formaldehyde formed. Rat livers were taken out and cytosolic fraction was prepared. GST activity was measured in cytosolic fraction by kinetics of thioether product formation over time. Results showed that in diabetic male and female SD rat hepatocytes, a significant ($p< 0.05$) increase in aminopyrine N-demethylase activity was observed only with 250µM mitragynine. For phase II drug metabolizing enzyme, mitragynine (0.25µM -250µM) significantly ($p<0.05$) inhibited the GST specific activity in both diabetic male and female rats. In conclusion this study indicates that the aminopyrine N-demethylase enzyme induction and GST activity inhibition by mitragynine is not influenced by sex. Aminopyrine N-demethylase activity increases only on high concentration of mitragynine and lower concentrations cannot be able to change it. However GST activity inhibition is dose-dependent.

Keywords: Mitragyna speciosa. Mitragynine. Rat hepatocytes. Aminopyrine N-demethylase. Glutathione (GSH). Cytosolic fraction.

Introduction

Herbal medicines are mixture of different plant's part like leaves, bark, root or fruit. These are widely used to treat health ailment since centuries in different traditions. In developing countries 80% people use herbal drugs for the treatment of inflammation, diabetes, colds, central nervous system disorder and cardiac disease [1]. Herbal remedies are supposed to be safer than therapeutic agents. However, possibility of herb–drug interactions increases when these are administered with drugs. The risk of interaction with the use of 2 products is 6% and with the use of 5 it becomes 50% [2]. Poorly absorbable herbs (rhubarb, flaxseed and aloe) can reduce the action of digoxin and warfarin. Adverse effect of warfarin and carbamazapine are increased when administered with meadowsweet and black willow. Corticosteroids metabolism reduced with use of licorice [3]. Pharmacokinetic drug interaction leads to induction and inhibition of enzyme system contributing in transportation, metabolism and excretion. Most common drug is warfarin and most common herb is St John's wort those caused reported herb-drug interaction [4]. The best example for this is the induction of CYP2B6 and CYP3A4 by St John's wort in liver. Number of studies were conducted to find possible herb drug N-demethylase interactions [5-7].

Mitragyna speciosa indigenous in Thailand, Malaysia and cultivated in subtropical and tropical regions of Asia. It is also found in Cambodia, East and West Africa and in India [8]. In Thailand it is called kratom and in Malaysia it is called ketum. In Thailand kratom leaves are chewed to increase work efficiency [9]. *Mitragyna speciosa* leaves stimulate the central nervous system and increase tolerance to hard work [10]. More than 25 alkaloids have been isolated from *Mitragyna speciosa* and major alkaloid is mitragynine. Mitragynine is an indole alkaloid and its structural formula is $C_{22}H_{31}O_5N$. Tsuchiya reported that mitragynine inhibits 2-deoxy-D-glucose- stimulated gastric acid secretion in rats through opioid receptors similar to morphine [11]. In addition, it also inhibits the guinea pig ileum by acting on opioid receptor and did not have any effect on smooth muscle contraction [12]. Moreover, chronic administration of mitragynine significantly impaired the cognitive function [13]. Grewal reported the pharmacology of mitragynine and it was being used to treat opium withdrawal [14]. He also reported its antihypertensive, antitussive and morphine like effects. Withdrawal symptoms & side effects from *Mitragyna speciosa* addiction are less intensive and life threatening than morphine [15]. Its pharmacological actions make it crucial to predict its interaction with prescribed drugs. There is no study available about the effect of mitragynine on drug metabolizing enzymes. Therefore following study is designed to evaluate the possible effect of mitragynine on phase I and phase II drug metabolizing enzymes, aminopyrine N-demethylase and glutathione S-transferase in diabetic rats.

There are two types of the reactions in drug metabolism mainly occur in liver called phase I and phase II reactions. In phase I reaction nonpolar molecules undergo hydrolysis, reduction or oxidation and converted into polar metabolites. These reactions are catalyzed by cytochrome P450 enzymes dominantly present in liver [16]. Phase II reactions are involved in conjugation of the organic compound with glucuronic acid, glycine, acetyl group, and sulphate or methyl group [17]. Conjugation reactions enzymes are uridine diphosphoglucuronosyl transferase (UGT), glutathione S-transferase, N-acetyl transferase (NAT) and sulphotransferaese (ST). Phase I and phase II enzymes play important role in the detoxification and excretion of xenobiotics [18].

Drug metabolism is influenced by gender, age and disease. Liver diseases significantly alter the activity of cytochrome P450. CYP1A2, 2C19 and 2E1 activities become less in liver disease [19]. Diabetes gets attention because it has become epidemics worldwide. In Malaysia 1.6 million adult diabetics is reported. Microvascular complications (retinopathy, neuropathy and microalbuminuria) and metabolic syndrome (obesity, hyperlipidaemia) are high in Malaysian diabetics including female [20].

Materials and Methods

Chemicals

All chemicals were used of analytical grade. Potassium dihydrogen orthophosphate was supplied by Ajax Chemicals Australia. Di-potassium hydrogen phosphate, tannic acid, calcium chloride, diethyl ether, barium hydroxide, potassium chloride, glucose monohydrate, potassium hydroxide, sodium hydrogen carbonate were purchased from R & M Chemicals, UK. Acetyl acetone, 1-chloro-2, 4-dinitrobenzene, sodium carbonate, streptozotocin, aminopyrine, glutathione reduced form, Bovine serum albumin, formaldehyde solution 37 percent, copper sulphate penta hydrate, collagenase type IV, Folin & Ciocalteu's phenol reagent, sodium potassium tartrate, and trypan blue were supplied by Sigma Chemicals Co, USA. Zinc sulphate and magnesium chloride supplied by HmbG Chemicals, UK. Potassium chloride was purchased from BDH Chemicals Ltd, UK. Tween 20 was purchased from Fisher Scientific, UK.

Mitragynine isolated from leaves of *Mitragyna speciosa* in the Center for Drug Research, Universiti Sains Malaysia, Penang Malaysia and was used in following experimental studies.

Experimental animals

Sprague- Dawley (SD) rats were taken from animal house of Universiti Sains Malaysia and kept in animal transition room of School of Pharmaceutical Sciences, USM until use. They were provided free access to Gold Coin chow and water *ad libitum*. Temperature was maintained at 25°C ± 2 and 12hours day & dark cycle. All experiments were done in control conditions. Male rats with body weight 200g – 250g and female rats with body weight 180g – 210g were selected. SD rats were divided into four groups.

1st Group Control male SD rat (n = 6)

2nd Group STZ induced diabetic male SD rat (n=12)

3rd Group Control female SD rat (n = 6)

4th Group STZ induced female SD rat (n = 12)

6 SD rats of each diabetic group (male & female) were used for N-demethylase activity and 6 SD rats for GST activity determination.

Solution of mitragynine: Mitragynine 1st dissolved in 1 ml 20% (v/v) Tween 20 then subsequently diluted with distilled water to form serial dilutions (0.0025 µM – 250 µM). Tween 20 is well tolerated in rats as measured in liver studies [21].

Induction of diabetes

SD rats were induced diabetes by using 60 mg/kg of streptozotocin (STZ) intraperitonally [22]. STZ was dissolved in ice-cold normal saline (0.9%NaCl) and injected immediately. Blood samples were collected from the tail of rats on third day after administration of STZ. Rats with blood glucose level higher than 15.6 mmol /L (at fasting state) was considered as type 1 diabetic rat.

Assay for aminopyrine N-demethylase activity

Hepatocytes Preparation

The rat was anaesthetized and liver was perfused with calcium free HBSS solution following collagenase buffer [23]. The liver was taken out and dispersed in incubation medium. Then suspension was filtered and filtrate centrifuged at 300 rpm at 10°C for 5 minutes. The cells were counted and viability was determined by trypan blue. Only cells more than 85% viability was used in further experiments. 6000 freshly isolated cells were used to study the effect of mitragynine on aminopyrine N-demethylase activity. Aminopyrine (25mM), incubation medium, hepatocytes (6 x 10^3 cells) and mitragynine (1.0ml) of different concentration (0.0025 µM -250µM) were added in petri dishes. Mitragynine was being replaced with distilled water in the control petri dish. Then these were incubated for 15 minutes on Belly Dancer® at room temperature. 25% (w/v) $ZnSO_4$ was added to stop the reaction after 5 minutes $Ba(OH)_2$ was added to neutralize $ZnSO_4$. The samples were transferred to test tubes and incubated in water bath shaker for 30 minutes at

60°C. According to colorimetric method of Nash [24] the quantity of formaldehyde formed was determined to measure aminopyrine N-demethylase activity. The N-demethylase activity was expressed as percent specific activity with respect to control.

Preparation of the rat liver cytosolic fraction

Rat's livers were taken out under CO_2 anesthesia. Livers were rinsed with ice cold water to remove the blood and then ice cold potassium phosphate buffer pH7.4. The livers were blotted dry, weighed and homogenized in three volume of 67 mM potassium phosphate buffer (pH 7.4) with 1.15% potassium chloride using a Potter-Elvehjem homogenizer. The livers homogenate were then centrifuged at 12,500 ^{x} g for 20 minutes at 4°C. The resultant supernatant was further centrifuged at 100,000 ^{x} g for 60 minutes at 4°C in an Optima ^{TM} TLX refrigerated ultracentrifuge (Beckman Coulter, Inc., USA). The supernatant represents cytosolic fraction, which was used for the analysis of GST enzyme activity. The liver cytosolic fractions were kept at -80°C until used.

GST Assay

Protein concentrations in cytosolic fractions of each group were determined by Lowry method [25]. Effect of mitragynine on GST activity was determined by method has been described by Habig [26] with slight modification. 0.125mg/ml final concentration of cytosolic protein was used with 30mM GSH & 30mM 1-chloro-2, 4-dinitrobenzene as a substrate in 100mM potassium phosphate buffer (pH 6.5). The mitragynine dissolved in1ml of 20 % (v/v) Tween 20 and further dilution was made with distilled water. The different concentrations of mitragynine range from 0.0025µM-250µM were tested for its GST specific activity. The absorbance of conjugated- substrate was read at 340nm on a Powerwave X340® for 5 minutes at room temperature. All GST assays were assessed in linearity conditions like incubation time and protein concentration. The GST activity was expressed as percent specific activity with respect to control. Tannic acid was used as positive control in all experiments.

Statistical Analysis

All results were expressed as mean ± standard deviation (SD) per experimental groups. Overall effect of the mitragynine was subjected to one- way ANOVA. Dunnett's test was used to evaluate the significance differences between the control and the experimental groups. Difference with a $P < 0.05$ was considered statistically significant. All analysis were performed using GraphPad Prism ® 5 Software

Result and discussions

Effect of mitragynine on aminopyrine N-demethylase activity

Mitragynine (250 µM) was shown to enhance significantly ($p<0.05$) N-demethylase activity 142% in diabetic male and 140% in female rat with respect to control. Mitragynine at concentrations 0.0025 µM - 25 µM could not induce significant change. (Fig. 1)

see Fig. 1

Effect of mitragynine on cytosolic GST activity

Mitragynine (0.25 µM - 250 µM) was shown to inhibit GST activity significantly ($p<0.05$) in male and female diabetic rats. It is shown in Fig.2 that there was dose - dependent GST activity inhibition (86% - 36.5%) in male rats. In female rats the GST inhibition (86.3% - 41%) was also dose dependent (Fig.3).

see Fig. 2

see Fig. 3

Mitragynine was showed more than 50% inhibition of glutathione S-transferase activity. The IC_{50} values were calculated on graph pad prism® 5 Software by plotting log concentration of mitragynine versus the percentage inhibition of GSTs specific activity. The IC_{50} value for diabetic rats of both male and female is 24.38 µM and 11.82 µM respectively. Taking a close look on the results it is assessed that N-demethylase activity induction and GST activity inhibition were more in diabetic male

rat than female rat.

Discussion

Freshly isolated hepatocytes have all co-factors and sub family of CYPs involved in drug metabolism. Therefore in vitro study resembles to *in vivo* situation. In addition, in vitro, CYP enzymes can be induced in the same manner as in vivo [27]. The important factors those influenced drug metabolism are gender, age and disease. In addition, drug metabolizing enzymes showed different activities in male and female. Isoenzymes CYP3A4 activity is twice in women than men. Similarly CYP2B6 and CYP2A6 showed more activity in female than male. CYP 2C11 is only expressed in male rats. Rest of the CYPs showed high activity in male. Our results are in agreement with this statement. There are widespread alterations in the CYP isozymes expression in diabetes [28]. There is marked induction in CYP1B1, CYP2B1, CYP1A2 and CYP2E1 isozymes however CYP2C11 decreased in diabetic rats. Among the hepatic cytochrome, CYP2C19, CYP2C8, CYP2D6, CYP2C18, CYP1A2 catalyzed the aminopyrine efficiently [29]. Taking into account aminopyrine is a suitable probe for determination of enzymatic inhibition / induction by the herbs or drugs. Our results showed that *in vitro* mitragynine would cause induction of cytochrome activity in diabetic male and female rat hepatocytes and may be due to increase expression of cytochrome P450 enzymes. It may enhance the metabolism of co administered drugs and may reduce the effectiveness of drug. However further experiments must be designed to evaluate the possible mechanism of increased N-demethylase activity and correlates the statement in vivo study.

GST enzymes play a role in detoxication of harmful chemicals, carcinogens and xenobiotics. Important substrates are polycyclic hydrocarbon epoxides formed by CYP450 in phase I metabolism and by-product of oxidative stress [30]. In diabetes, oxidative stress become more worsens by induction of CYP2E1 [31]. Hepatic glutathione content and GST activity markedly decrease (65%) in diabetic rats [32]. In vitro study results showed that the mitragynine produced dose - dependent inhibition of cytosolic GST activity in both diabetic male and female SD rat. Various anticancer drugs are being detoxified by GST [33] and GST level increase during continuous chemotherapy. Over expression of GSTs in cells causes resistance to alkylating agent [34]. Plants those exhibit GST inhibition activity may have capability to enhance the efficiency of chemotherapeutic agent [35]. The mechanism of alteration the GST activity by mitragynine is yet to be discovered and future experiments will be conducted to correlate our findings *in vivo* study.

Conclusion

In vitro mitragynine at the concentration of 250µM is exerted the enhancement of aminopyrine metabolism by aminopyrine N-demethylase in diabetic male and female SD rats. Mitragynine showed significant inhibition in both male and female diabetic SD rats. However this is an *in vitro* study and future studies are necessary to confirm this statement via determination of molecular mechanism and *in vivo* experiments.

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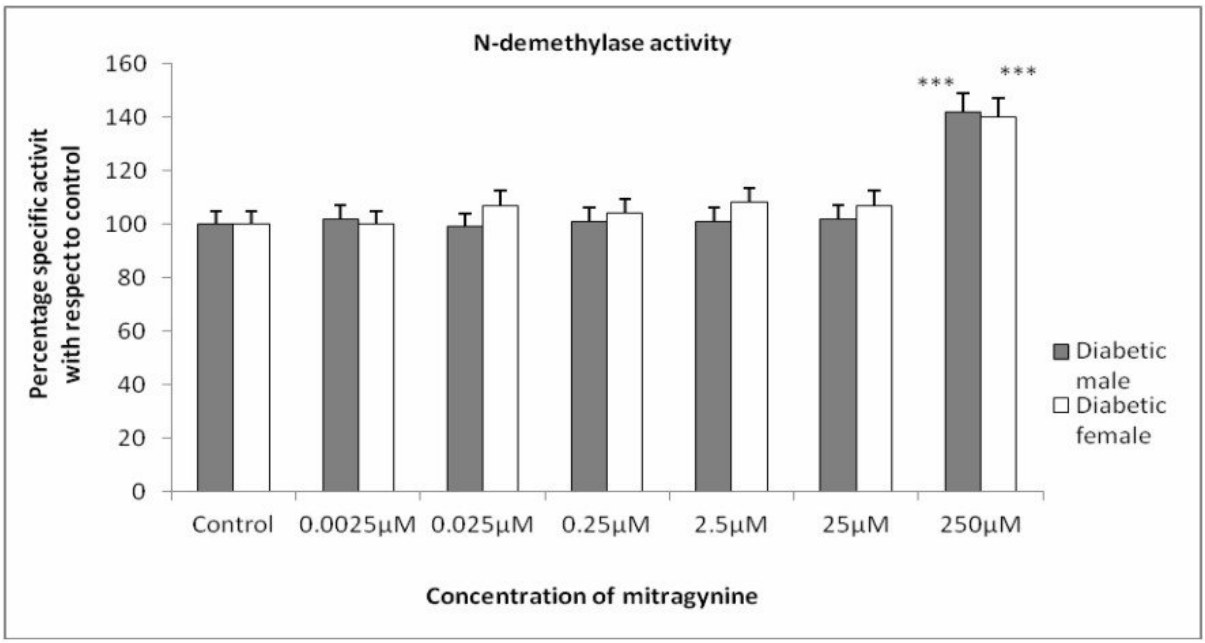

Fig. 1: Effect of mitragynine on aminopyrine N-demethylase activity in diabetic male and female rat hepatocytes (mean± SD; n = 6; ***p< 0.001 using one-way ANOVA).

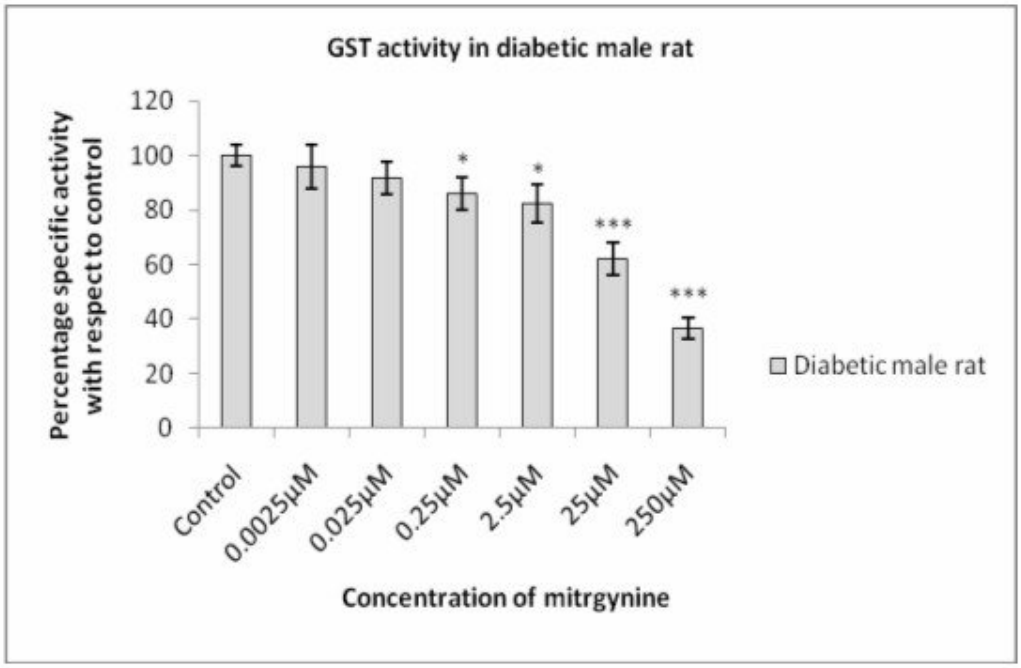

Fig. 2: Effect of mitragynine on GST specific activity in cytosolic fraction of the diabetic male rat (mean ± SD; *p< 0.05; ***p< 0.001; using one-way ANOVA).

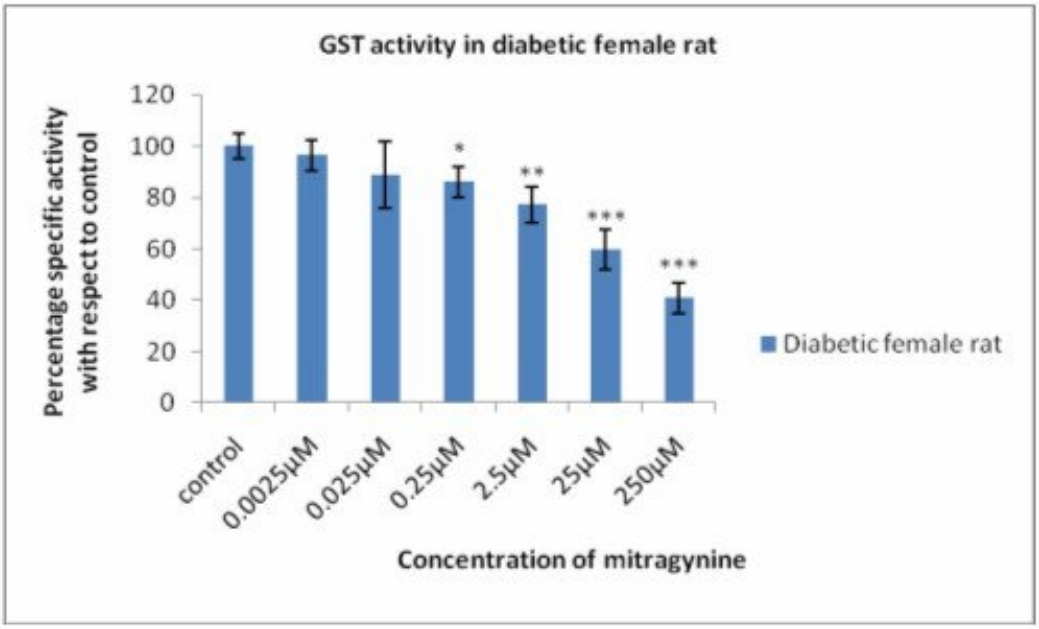

Fig. 3: Effect of mitragynine on GST specific activity in cytosolic fraction of the diabetic female rat (mean ± SD; *p< 0.05; **p < 0.01; ***p< 0.001; using one-way ANOVA).