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African Journal of Pharmacy and Pharmacology

Review

Review on pharmacological activities of liriodenine

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This review describes the pharmacological properties of liriodenine. This alkaloid has been isolated from plant species of many genera and exhibits a wide range of pharmacological activities that have been reported. This present paper enumerates an overview of pharmacological aspects that are useful to researchers for further exploration in order to develop the potential of this alkaloid.

Key words: Liriodenine, alkaloid, pharmacological activities, *Liriodendron tulipifera* L.

INTRODUCTION

Liriodenine (8*H*-benzo[γ]-1,3-benzodioxo[6,5,4-en]-quinolin-8-one) is an oxoaporphine alkaloid (Figure 1). This alkaloid was isolated for the first time from *Liriodendron tulipifera* L., and was subsequently isolated from plant species of many genera (Warthen et al., 1969), mainly found in the families of Magnoliaceae, Annonaceae, Rutaceae, Monimiaceae, and Menispermaceae (Bentley, 2001; Hsieh et al., 2005; Lan et al., 2003; Lin et al., 1994; Nissanka et al., 2001; Woo et al., 1997; Wu et al., 1990). This has a wide range of pharmacological activities, such as activity against Gram-positive bacteria (Camacho et al., 2000; Chang et al., 2004; Li et al., 2009; Mohamed et al., 2010; Rahman et al., 2005; Waechter et al., 1999), antifungal (Hufford et al., 1980; Khan et al., 2002), antitumoral (Chen et al., 2009, 2012; Liu et al., 2009), antiarrhythmic activity (Chang et al., 1996, 2001; Chung et al., 2004), antiviral activities (Mohamed et al., 2010) and antiplatelet actions (Chen et al., 1997; Pyo et al., 2003). The objective of this work was to compile information about liriodenine, which may help researcher to understand the efficacy and potency of this alkaloid.

PHARMACOLOGICAL ACTIVITIES

Antimicrobial and antifungal properties

Liriodenine, was identified as a potentially useful antimicrobial antibiotic against *Staphylococcus aureus, Mycobacterium smegmatis, Candida albicans* and *Aspergillus niger* (Hufford et al., 1975, 1980). Mice were injected with a lethal dose of *C. albicans* NIH B311 and were administered varying doses of liriodenine. Reductions in the number of colony-forming units (CFU) measured per milligram of kidney tissue were observed in drug-treated animals compared to vehicle-treated control mice (Clark et al., 1987). The IC_{50}/minimum inhibitory concentration values of liriodenine against *C. albicans*, *Cryptococcus neoformans*, *S. aureus*, and Methicillin-resistant *S. aureus* (MRS) were 3.5/6.25, 2.0/12.5, 2.0/3.13, and 2.0/3.13 µg/ml, respectively (Zhang et al., 2002).It has recently been reported that liriodenine has great potential as an environmental benign wood preservative (Wu et al., 2012). Thus, it is effective against the white-rot fungi *Lenzites betulina* and *Trametes*

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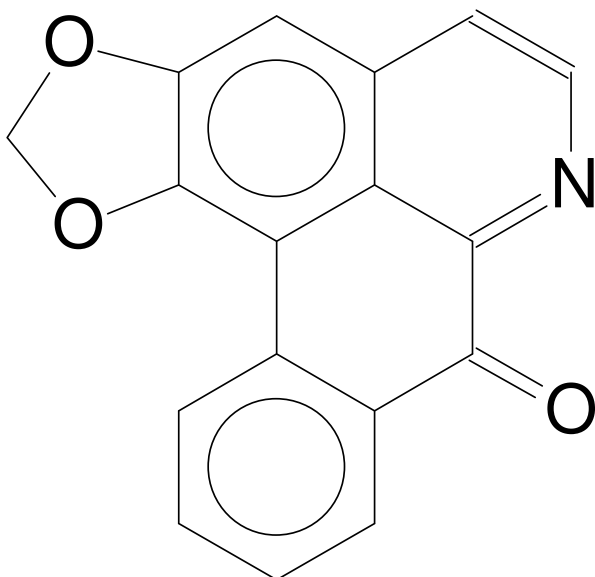

Figure 1. Structure of liriodenine.

versicolor and the brown-rot fungi *Laetiporus sulphureus, Gloeophyllum trabeum,* and *Fomitopsis pinicola*, suggesting effectively inhibiting the growth of wood-rotting fungi.

Antitumor activity

Owing to planar aromatic structure, the antitumor activities of liriodenine can be primarily attributed to intercalate between the neighboring base pairs of the DNA double helix (Woo et al., 1997). The importances of the oxo function induce cytotoxicity as well as inhibitory effects on precursor incorporation into DNA (Tzeng et al., 1990). Moreover, liriodenine also catalytically inhibits topoisomerase II to block DNA synthesis inducing cell cycle G1 arrest and increases tumor suppressor p53 and inducible nitric oxide synthase expression (Chang et al., 2004; Hsieh et al., 2005; Wu et al., 1990). Meanwhile, liriodenine has been shown to raise induced oxide nitric synthase (iNOS) expression and oxide nitric (NO) production, followed by increasing p53 production, and then up-regulating the p21 and p27 expressions (Chen et al., 2012). These events decreased the expressions of cyclin D1 and cyclin-dependent kinase, followed by reducing the pRb phosphorylation (ppRb), and finally triggering the G1/S arrest of cell cycle on human SW480 colon cancer cells (Chen et al., 2012). Furthermore, liriodenine has been demonstrated to block cell cycle progression at the G2/M phase by reduction of G1 cyclin D1, accumulation of G2 cyclin B1 and the decrease of enzymatic activity of the cyclin B1/cyclin-dependent kinase 1 complex in human A549 lung cancer cells (Chang et al., 2004). Liriodenine, isolated from the roots of *Cyathostemma argenteum*, has been reported to exhibit moderate cytotoxic activity against breast cancer cell lines (Khamis et al., 2004). The anti-cell activity of liriodenine from *Liriodendron tulipifera* has been shown to have effects on human melanoma A375.S2 cells (Chiu et al., 2012). Cell growth inhibition activity of liriodenine demonstrated a potent cytotoxicity against KB, A-549, HCT-8, P-388 and L-1210 cells with IC_{50} values of 3.6, 2.6, 2.5, 2.1, and 8.5 μM, respectively (Guo et al., 2005).

Cardiovascular effects

Liriodenine at the dose of 10^{-6} g/kg diminished the left ventricular end-systolic elastance E_{es} and effective arterial elastance E_a (Chang et al., 2001). The *in vivo* biological studies indicated that through inhibition of the Na^+ and I_{to} channels, liriodenine suppresses antiarrhythmic activity induced by myocardial ischaemia reperfusion (Chang et al., 1996). An increased concentration of NO limits myocardial ischemia-reperfusion injury. Liriodenine has been shown to reduce the extent of cardiovascular injuries under ischemia-reperfusion conditions by preserving the eNOS and the NO production (Chang et al., 2004). The transient coronary artery occlusion often leads to malignant ventricular arrhythmias during the ischaemic and reperfusion periods (Ferrier et al., 1985). Therefore, liriodenine may provide a satisfactory therapeutic potential in the treatment of cardiac arrhythmias.

Antiplatelet actions

Liriodenine inhibited the contractile responses of guinea-pig trachea and acted as a M3 receptor antagonist in paced left as well as in spontaneously heartbeat right atria of guinea-pigs (Lin et al., 1994). Moreover, liriodenine abolished adenosine diphosphate (ADP)-induced aggregation activity in human whole blood (Moharam et al., 2010) and on washed rabbit platelets (Chen et al., 1997).

Medicinal inorganic chemistry properties

Since the success of *cis*-platin and related platinum complexes as anticancer agents, developing other active transition metal anticancer complexes with better efficiency and new mechanisms of action has attracted many bioinorganic chemists' interest and has became one of the focused research fields of bioinorganic chemistry (Liu et al., 2009). Based on the planar character and N-7/O-8 electron donor sites, liriodenine has the coordination capacity by forming chelates with several metal ions such as metal ions to form metal-based function bifunctional compounds with potential synergistic effects on antitumor activity.

Liriodenine (L) reacted with Mn(II), Fe(II), Co(II) and Zn(II) to afford four metal complexes: $[MnCl_2(L)_2]$, $[FeCl_2(L)_2]$, $[Co(L)_2(H2O)_2\ Co(L)_2(CH3CH2OH)_2](ClO4)_4$, and $[Zn_2(L)_2(m2\text{-}Cl)_2Cl_2]$, all complexes bind more intensively to the DNA helix than does liriodenine and effectively inhibit topoisomerase I even at a low concentration (≤10 µM) (Liu et al., 2009). Furthermore, Pt(II) and Ru(II) were chosen for the synthesis of metal complexes with liriodenine as ligand. The antitumoral activities against a series of human tumor cell lines *in vitro* showed that these complexes exhibit higher antitumor activities than liriodenine or cisplatin does (Chen et al., 2009). These results demonstrated that the metal complexes of planar liriodenine reinforce the DNA-binding ability. Beside two-metal ion, liriodenine was used as a bioactive ligand to react with gold(III) compounds. The *in vitro* cytotoxicity towards five human tumor cell lines showed that antiproliferative properties of the complex are compared with free liriodenine, with IC_{50} values falling in the 2 to 16 µM range. The complex induced an S-phase arrest and significantly inhibits topoisomerase I *in vitro* at low concentration (≥ 25 µM or lower) (Chen et al., 2012). Liriodenine metal complexes may offer a new effective strategy to achieve higher cytotoxic activities.

Central nervous system activities

Liriodenine is reported as a sedative of the central nervous system (Rios et al., 1989). Furthermore, liriodenine has been shown to regulate dopamine biosynthesis by partially reducing tyrosine hydroxylase (TH) activity and TH gene expression and has protective effects against L-DOPA-induced cytotoxicity in PC12 cells (Jin et al., 2007). It is suggested that the isoquinoline ring planarity play a key role in the inhibition of dopamine.

CONCLUSION

This review provides the multiple biology effects of liriodenine which made it a valuable potential of molecule. The pharmacological aspects of liriodenine have been studied extensively; however, this alkaloid has not yet been developed as a drug. Ongoing and detailed research is required for the identification, cataloging and documentation of this alkaloid, which may provide scientific and encourage development of this alkaloid for pharmaceutical uses or therapeutic uses.

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Review

Evaluation of hydro-alcoholic extract of leaves of *Boerhaavia diffusa* for anxiolytic activity in rats

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The hydro-alcoholic extract of *Boerhaavia diffusa* of leaves was investigated for evaluation of exhibited anxiolytic activity in rats at a dose 100 and 200 mg/kg by p.o. route for elevated plus maze test, hole-board test, haloperidol induced catalepsy and ketamine induced sleep method. Results of *in vivo* activity lead to the conclusion that the hydro-alcoholic of *B. diffusa* showed predominantly significant activity which is compared to the standard drug, diazepam (0.5 mg/kg).

Key words: *Boerhaavia diffusa*, anxiolytic activity, diazepam, haloperidol, hydro-alcoholic extract, ketamine, sleep.

INTRODUCTION

Boerhaavia diffusa family: Nyctaginaceae, Sanskrit: "Punarnava is a perennial creeping weed found through-out India. The leaves of *B. diffusa* are reported for their use in the indigenous system of medicine for the treatment of dyspepsia (Table 1), jaundice, enlargement of the spleen and abdominal pain (Kirtikar and Basu, 1956). A decoction of whole plant is taken with milk in the early morning to cure jaundice and weakness However, no scientific evaluation of these claims appears to have been undertaken so far. In the present study, we were made to validate the folklore use of this plant as anxiolytic activity.

MATERIALS AND METHODS

Plant

The leaves of *B. diffusa* were collected in August, 2009 from local market of Bopal, India and were authenticated. The voucher specimen (NIPS/PC/105) was preserved in the laboratory for reference.

Preparation of extract

The leaves were dried under shade, powdered and passed through 40 meshes, and were stored in closed vessel for further use. *B. diffusa* was extracted using solvent system of 70% methanol and 30% water in soxlate apparatus at a temperature of 40 to 60°C. On the seventh day, 35 cycles of the soxlate extraction were done. The extract was filtered and concentrated in a vacuum under pressure using rotary flash evaporator.

Phytochemical analysis of the extract

The extract was screened for the presence of various constituents employing standard screening test (Trease and Evans, 1985). Conventional protocol for detecting the presence of glycosides, saponins, flavonoids, tannins, etc., was used. Several phytoconstituents like flavonoids and saponin are known to have anxiolytic activity (Ambavade et al., 2006; Nayak et al., 2004).

Toxicity studies

Toxicity studies of hydro-alcoholic extract were carried out in oral doses of 1000 to 2000 mg/kg body weight using albino rats. After test extract administration, animals were observed for 72 h period. The number of deaths was expressed as a percentile and the LD_{50} was determined by probate test using the death percentage versus the log dose (Turner RA.1965). Study protocol was approved from the Institutional Animal Ethics Committee (IAEC).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM), and statistical difference was analyzed using Dunnet t test

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Table 1. Treatment schedule.

Group	Treatment	Dosage, route of administration
1	Distilled water (vehicle)	1 ml/kg, p.o
2	Hydro-alcoholic extract	100 mg/kg, p.o
3	Hydro-alcoholic extract	200 mg/kg, p.o.
4	Diazepam	0.5 mg/kg, i.p

n = number of animals used in each group and treatment duration (10 days).

Table 2. Effect of *B. diffusa* extract on time spent in open arm.

Treatment	Time spent in open arm
Vehicle	29.5 ± 4.21
Extract 100 mg/kg	77.0 ± 15.19*
Extract 200 mg/kg	45.5 ± 7.3
Diazepam 0.5 mg/kg	70.25 ± 1.6*

Values are mean ± SEM of observation. F_{12} = 6.38, P = 0.008; compare to respective vehicle control group. *Represents data that is significant.

and results were considered significant when P < 0.05.

EVALUATION OF ANXIOLYTIC ACTIVITY

Animals

Adult albino rats (150 to 200 g) were used in this study. They were housed in well ventilated rooms (temperature 22 ± 2°C, humidity 65 to 70% and 12 h light/dark cycle) and fed with standard rodent pellet diet (Lipton India Ltd., Bombay) with tap water *ad libitum*.

Elevated plus maze model

Elevated plus maze (EPM) test (Pellow et al., 1985) for studying the anxiolytic effect in rodents was used. EPM consists of two open arms (15 × 10 cm) and two closed arms (50 × 10 × 40 cm) with an open roof elevated at 50 cm. 1 h after the oral administration of drugs, the rat was placed at the centre of the maze, facing one closed arm. During a 5 min test period, the following measures were taken: the time spent in the open and closed arms; and total number of arm entries. The duration of treatment was 10 days. The result is as shown in Table 2.

Hole-board test

Placing a rat on the hole-board apparatus, elevated to 45 cm from the table, induced anxiety in it as it was exposed to a new environment. The anxiogenic agents reduce the number of head poking, whereas the anxiolytic agents increased the number of head poking.

The hole-board apparatus consist of metal plate floor (40 × 40 cm) placed at 25 cm above the ground. The metal plate consist of six hole (1.5 cm in diameter) spaced symmetrically in a diamond pattern. A rat was placed on one corner of the apparatus and was observed for the next 5 min for the number of head poking (Mohan et al., 2005). The results are as shown in Table 3.

Haloperidol induced catalepsy

Purpose and rational catalepsy in rats was defined as a failure to correct an externally imposed unusual posture over a prolonged period of time. Neuroleptics which have an inhibitory action of nigrostriatal dopamine system induced catalepsy (Costall and Naylor, 1974; Chermat and Simon, 1975) while neuroleptics with title or no nigrostriatal blockade produce relatively title or no cataleptic behavior. Furthermore, cataleptic symptoms caused Parkinson-like extrapyramidal side effects in rodents seen clinically with administration of antipsychotic drugs (Duvoisin, 1976).

Procedure

Albino rats were divided into four groups each. They were administered vehicle, that is, water by intraperitoneal (i.p) route. After 30 min, the rats were administered haloperidol 1 mg/kg i.p and both forepaws of rats were place on a wooden bar elevated 6 cm above the ground. The duration for which the rats retains the fore paws on the elevated bar was noted at 0, 30, 60, 90 and 120 min. The cut off time was 300 s.

The two doses of the extract (*B. diffusa*), 100 and 200 mg/kg for animals in each group, were administered to both groups: the 2nd group was given 100 mg/kg and the 3rd group was given 200 mg/kg p.o. After 45 min, the same group of animals was treated/ administered with haloperidol (1 mg/kg i.p) and both forepaws of rats were placed on wooden bar elevated 6 cm above the ground. The duration for which the rats retain the forepaws on the elevated bar was noted at 0, 30, 60, 90 and 120 min, though the cut off time was 300 s. The results are as shown in Table 4.

Ketamine induced sleep

Materials required are ketamine (80mg/kg), extract (dose A and B), syringe (1 ml), animals and vehicle (distilled water). The animals were divided into four groups of similar body weight of same sex, each groups were treated separately with calculated dose. Animal was treated with either vehicle or extract (p.o) and after 45 min, the animals are administered ketamine (80 mg/kg i.p), then the animals were placed in the observation table for 1 h induction of sleep and the duration of sleep was recorded (Winters et al., 1972). The results are as shown in Table 5.

RESULTS AND DISCUSSION

Elevated plus maze (EPM) model

In the animal study, we found out that *B. diffusa* plant extract significantly reduced the anxiety; the result is as shown in the Table 2. Two doses of extract were taken and observed in elevated plus maze apparatus. The

Table 3. Effect of extract on the number of head poking in hole board apparatus.

Treatment	No. of head pocking
Vehicle	5.0 ± 0.9
Extract (100 mg/kg)	7.75 ± 0.85
Extract (200 mg/kg)	6.25 ± 1.1
Diazepam (0.5 mg/kg)	7.75 ± 0.62

Values are mean ± SEM. F_{12} = 1.88, P = 0.188; compared to respective vehicle group control. Data that is not significant.

Table 4. Effect of ketamine on duration of sleep.

Treatment group	Duration of sleep
Vehicle (1 mg/kg) + ketamine (80 mg/kg i.p.)	62 ± 1
Extract (100 mg/kg) + ketamine (80 mg/kg i.p.)	54 ± 1.95*
Extract (200 mg/kg) + ketamine (80 mg/kg i.p.)	51.25 ± 2.49*

Values are mean± SEM of observations. P = 0.009; compare to respective vehicle group control. *Represents data that is significant.

Table 5. Interaction of the extract with haloperidol at 0, 30, 60 and 120 min.

Treatment	0 min (mean±SEM)	30 min (mean±SEM)	60 min (mean±SEM)	90 min (mean±SEM)	120 min (mean±SEM)
Vehicle + Haloperidol (1 mg/kg)	0 ± 0	255 ± 44.25	300 ± 0	300 ± 0	83.5 ± 0.5
Extract 100 mg/kg + Haloperidol (1 mg/kg)	0 ± 0	0 ± 0*	180 ± 9.25*	195 ± 61.53*	255 ± 28.2*
Extract 200 mg/kg + Haloperidol (1 mg/kg)	0 ± 0	0 ± 0*	0 ± 0*	0 ± 0*	16.25 ± 2.39*

Values are mean ± SEM of 5 observations. *Represents that data is significant. All results were analyzed by dunnet t test and results were considered. significant when p<0.05.

doses of extract were compared to the control group and standard diazepam (0.5 mg/kg i.p). According to the result, the low dose that is, 1st extract was found significant as compare to the high dose of 2nd extract and the value of "p" was found (P = 0.008). Elevated plus maze was used to observe the anti anxiety activity of the extract on the rats, as the dose of the extract was compared to the time spent by the vehicle (100 mg/kg) in treating rats, which shows that the extract has anxiolytic activity.

Hole-board test

Two doses of the extract were taken and observed in hole-board apparatus. The dose of the extract was compared to the control group and standard diazepam (0.5 mg/kg i.p). According to the result, both doses were found not significant and the value of "p" was found (P = 0.188).

Effect on sleep duration using ketamine

The two doses of the extract + ketamine (80 mg/kg) were taken as stated previously and the

duration and latency of sleep were observed. The two doses of the extract were compared to the control group, and the result is as shown in Table 4. According to the result, both doses of the extract significantly reduced the duration of sleep in rats. Both doses of the extract were found significant at P = 0.009.

Drug interaction with centrally acting drug

Haloperidol is a potent neuroleptics drug which induces catalepsy in rats. Two doses of the extract were taken for experiments as stated in the materials and method and the result is as shown in Table 5. The two doses of the extract were compared to the vehicle treated group, in different time interval or duration of time like 0, 30, 60, 90 and 120 min. In 0 min, both doses of the extract were not significant, but in 30 min duration, both they were found significant (P = 0.001). In 60 min, the extract dose of 100 mg/kg was found significant (P = 0.001) and the extract dose of 200 mg/kg was also found significant. In 90 min, both doses of the extract were found significant (P = 0.001) and in 120 min, it was found that both doses of the extract was found significant, that means the extract in maximum time interval decreases catalepsy with the haloperidol drug that acted similarly like the agent which is dopaminergic or which is working as a D_2 receptor agonist.

Conclusion

In the present study, we used the EPM model of anxiety to evaluate the anxiolytic effects of the hydro-alcoholic extract of *B. diffusa*. The elevated plus maze is currently one of the most widely used models of animal anxiety (Hoggs, 1996). The extracts of *B. diffusa* increased the time spent in open sided arms of the plus-maze by rat in the dose range of 100 mg/kg. Maximum activity by all the extracts were produced at 100 mg/kg and the response was reverted when the doses were increased to 200 mg/kg. Plants containing sterols, flavonoids, etc., were reported to have anxiolytic activity (Ambavade et al., 2006; Nayak et al., 2004) and preliminary phytochemical screening revealed the presence of sterols, tannins and flavonoids in the *B. diffusa*. Therefore, the anxiolytic activity of *B. diffusa* may be due to the presence of tannins, sterols, flavonoids, etc. However, further investigations are required to isolate the phytoconstituents responsible for anxiolytic activity and to find their mechanism of action.

As expected, diazepam produced significant increases in open arm time and in a number of entries into the open arms. Diazepam also increased the total number of entries. These data are in agreement with the results of other studies, where diazepam and other benzodiazepines have been shown to produce robust anxiolytic effects in a variety of anxiolytic screening procedures, including conflict model (Vogel et al., 1971), EPM procedures (Pellow et al., 1986) and other non punishment procedures (Winslow et al., 1991). The result of the present study suggests that hydro-alcoholic extract of *B. diffusa* plant may possess significant anxiolytic activity.

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Full Length Research Paper

Pharmacists' expectations regarding the services provided by drug and poison information center

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Health care professionals have been surveyed concerning their satisfaction with Drug and Poison Information Centers' (DPICs) services, but have rarely been questioned regarding their expectations. Knowing these expectations would be the starting point for improvement of DPIC's services. As pharmacists are a major group of DPICs' clients, this study was aimed to clarify the expectations of Iranian pharmacists regarding the services provided by the DPIC in Tehran University of Medical Sciences. Data was collected from questionnaire which was distributed among 562 pharmacists in August, 2008. Data was analyzed by producing descriptive statistics using Statistical Package for Social Sciences (SPSS) software (version 17). The mean importance rating for each expectation was determined and sorted in descending order. A response rate of 87.9% was achieved. Drug information textbooks and medical reference books were the 'most commonly used' drug information resources. Thirty six expectations had a mean importance rating of ≥ 3 (3 = important). Currently, there is a good fit between Iranian pharmacists' expectations and DPIC services provided. This survey identified several areas where services was expected but not provided (e.g. provision of telephone consultation services 24 h a day). In instances, where there was a gap between expectations and services provided, the DPIC made programmatic changes to align service with expectation when feasible.

Key words: Drug and poison information center (DPIC), pharmacists, expectations.

INTRODUCTION

A Drug and Poison Information Center (DPIC) is a formal unit with a series of resources and a professionally trained team engaged in providing accurate and factual information about drugs and poisons (Hall et al., 2006; George and Rao, 2005). World's DPICs are mainly affiliated to hospitals, faculties of pharmacy, faculties of medicine and other related organizations (Mullerova and Vlcek, 1998). The provision of accurate, prompt, timely and evidence-based drug and poison information to health care professionals is an important mechanism to promote safe and effective drug therapy for patients (Lakshmi et al., 2003; Ngo Su-Yin and Ponampalam, 2009). The complexity of the current drug literature has made the selection and use of correct drugs in an appropriate manner, a challenging task (Asiri et al.,2007). Lack of unbiased drug information is one of the factors that make the health care providers unable to update their knowledge about drugs which results in an increasing demand for drug information to promote patient care. Therefore, DPICs' services are necessary to provide support for health care providers (George and Rao, 2005; Wongpoowarak et al., 2010; Rosenberg, 1983).

The first drug information center (DIC), was established in 1962 at the University of Kentucky. Since then, DICs have expanded outside academic settings to include hospitals and pharmaceutical industry (Dada et

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Table 1. Demographic variables of survey respondents.

Demographic variable		Frequency n (%)
Gender	Male	201 (40.6)
	Female	291 (58.8)
Pharmacy practice experience (years)	< 5	129 (26.1)
	Between 5-10	86 (17.4)
	Between 10-15	108 (21.8)
	>15	162 (32.7)
Practice field	Hospital pharmacy	49 (9.9)
	Community Pharmacy	353 (71.3)
	Pharmaceutical Industry	32 (6.5)
	Others	47 (9.5)
Being familiar with DPICs' activities and services	Yes	224 (45.3)
	No	228 (46.1)
Estimated number of contacts with DPICs during last month	No contact	373 (75.4)
	< 5	60 (12.1)
	< 10	8 (1.6)
	> 10	5 (1.0)

al., 2008). Iran's first DPIC was opened in Tehran Ministry of Health, Under-secretary of Food and Drugs in February, 1997 with the intention of providing a centralized resource center for drug and poison emergency treatment advice and information to healthcare professionals, industries and general public (Nikfar et al., 2000). Health care professionals have been surveyed concerning their satisfaction with DPICs services, but have rarely been questioned regarding their expectations (Rheney et al., 2000). As the health care providers expect guidance and expert advice from DPICs', knowing client's expectations would be the starting point for improvement of DPICs' services (Scala et al., 2001). Pharmacists are one large group of DPICs' clients. This study was conducted to clarify the expectations of Iranian pharmacists regarding the services provided by the DPIC in Tehran University of Medical Sciences which was established in 2007.

METHODOLOGY

The study was descriptive. A questionnaire was prepared to investigate the pharmacists' expectations about DPICs. We invited all 562 pharmacists in Iranian Pharmacists Association Annual Congress which was held in August 2008 to participate in this survey.

A cover, describing the world's DPICs background and activities was attached to the questionnaire. The names of the respondents were not requested to maintain anonymity. Questions in this study covered a number of areas as follows:

1. The first section included the survey population demographic information including age, sex, professional or educational qualifications, university degree and job experience.
2. The current sources of drug and poison information being utilized by respondents were elicited from a list of suggested options (drug and poison information centers, textbooks, software's, journals and websites and colleagues) and the frequency of the sources usage was ranked to the following three levels: almost never, occasionally and frequently.
3. Thirty seven expectations were developed in five choices Likert-Scale. Survey instructions directed the respondents to rank the importance of each expectation on a scale of 1 to 5, where 1 = not important, 2 = somewhat important, 3 = important, 4 = very important and 5 = extremely important.

Statistical analysis

The filled questionnaires were analyzed by producing descriptive analysis using the Statistical Package for Social Sciences (SPSS for MS windows version 17). The mean importance rating for each expectation was determined and sorted in descending order.

RESULTS

Demographics

Out of the 562 potential responders who received the questionnaires, 494 fulfilled them, giving a response rate around eighty eight percent (87.9%). The average age of the respondents was around 41 years (41.49 ± 12.61). 95.1% of the pharmacists surveyed had a PharmD degree. The demographics of the responders are summarized in Table 1 (Note that the percentages through

Table 2. Frequency of using each source of drug information.

Variable	Frequency (%)
Using DPICs	
Almost never	175 (35.4)
Occasionally	176 (35.6)
Frequently	16 (3.2)
Using drug information textbooks and medical reference books	
Almost never	23 (4.6)
Occasionally	157 (31.7)
Frequently	250 (50.5)
Using drug information software's	
Almost never	94 (19.0)
Occasionally	179 (36.2)
Frequently	75 (15.2)
Using medical and pharmaceutical journals	
Almost never	36 (7.3)
Occasionally	232 (46.9)
Frequently	147 (29.7)
Using drug information websites	
Almost never	84 (17.0)
Occasionally	180 (36.4)
Frequently	98 (19.8)
Consulting expert colleagues	
Almost never	42 (8.5)
Occasionally	230 (46.5)
Frequently	109 (22.0)

this manuscript are all computed considering the number of missing cases in questionnaires).

The main resources used by responders for drug information are listed in Table 2. Tertiary sources, such as 'drug information textbooks and medical reference books' followed by 'medical and pharmaceutical journals and 'expert colleagues' formed the large bulk of the frequently used drug information resources for answering the queries (Table 2).

There were 36 expectations included on the survey instrument. Expectations are presented in the order of descending mean importance rating in Table 3. Thirty six (97%) survey expectations had a mean importance rating of ≥3 (3 = important). 'The provision of new drug information' and 'the provision of information on radiopharmaceutical and chemotherapeutic drugs preparations' had the most and the least importance ratings among all expectations, respectively.

The respondents' acquaintance with the DPIC's services and their future approach to make phone calls with these centers are summarized in Table 4.

DISCUSSION

DPICs are established with the primary goal of providing information to health care providers in need of medication-related issues (Sawalha, 2008). Expectation surveys are being used to improve the match between services provided and the client expectations (Karen et al., 2000). Comprehensive reviews of DPICs' services have been reported, but relatively little is known of the expectations of health care professionals regarding the services of these centers. We surveyed pharmacists, because they frequently use the services of the DPICs, so, constituting the major class enquirers for these centers.

Our survey data indicate that there is a good match between our pharmacists' expectations and services provided by DPIC. Most of the expectations with the highest importance ratings are the center's basic functions, like 'provision of new drug information', 'provision of drug use during pregnancy information' and 'provision of drug-drug and drug-food interaction information'. Similar to our

Table 3. Importance ratings for pharmacists' expectations.

Expectation	Mean rating	Standard deviation
Provide new drug information	4.31	0.89
Provide telephone consultation services 24 hours a day	4.13	0.95
Provide drug use during pregnancy information	4.08	0.93
Provide drug-drug and drug-food interaction information	4.06	0.90
Provide pediatric drug dosing information	3.96	1.00
Provide continuing education courses about drug information for health care professionals	3.94	0.95
Provide drug use during lactation information	3.89	0.99
Provide treatment information for toxic ingestions	3.82	0.97
Provide information on drugs' adverse drug reactions	3.78	0.98
Provide foreign drug identification	3.73	1.02
Provide drug dosing information	3.65	1.04
Provide cleaning substances hazards information	3.62	1.04
Provide other referral services (i.e., if the center does not have the information, refer the caller to where the information is available)	3.60	0.98
Provide consultation for health-care providers regarding poisoning	3.54	1.02
Provide clinical signs and symptoms information regarding toxic ingestions	3.50	1.03
Provide pesticides hazard information	3.49	1.04
Provide carbon monoxide hazards information	3.47	1.07
Provide drug storage condition information	3.44	1.03
Provide smoke inhalation hazards information	3.44	1.03
Provide drug dosing information in special patients	3.41	1.11
Research information from manufacturers for various products	3.41	1.03
Education/ training of center's health care professionals	3.39	1.03
Provide patient-specific recommendations in electronic/ written format	3.37	1.44
Provide printed material from our library of information including fax services	3.37	1.08
Provide insect stings hazards information	3.35	1.01
Collect and provide demographic/epidemiology information (e.g., regarding poisonings, trends in overdoses of street drugs)	3.34	1.01
Referral of the general public to physician when needed	3.32	0.99
Provide education of the general public regarding poisoning	3.32	0.99
Provide dietary supplement information	3.30	2.23
Provide chemical spills and industrial exposures hazards information	3.30	1.08
Identify ingredients of brand-name products	3.21	1.06
Provide information on herbal drugs	3.14	1.07
Provide green chile dermal exposure hazards information	3.12	1.05
Provide rattlesnake bites hazards information	3.11	1.13
Provide identification of tablets or capsules	3.06	1.04
Provide information on radiopharmaceutical and chemotherapeutic drugs preparations	2.88	1.19

Table 4. Pharmacists' future approach towards using DPICs.

Future approach	Frequency (%)
Previous acquaintance/Future phone calls	225 (45.5)
Previous acquaintance/No future phone calls	2 (0.4)
No Previous acquaintance/Future phone calls	161 (32.6)
No Previous acquaintance/ No future phone calls	1 (0.2)

findings, 69% of the respondents in a study by Rosenberg et al. (2009) indicated that the primary mission of the drug information centers is 'the provision of not-for-profit new drug information services'. In addition, the most frequent type of inquiries in a drug information center at the University of Kansas Medical Center involves information on these subjects: therapeutic use, dosage, product identification and adverse drug reactions (Pradhan, 2002). Also, the majority of inquiries which are most frequently asked in Food and Drug Administration (FDA) DPIC are related to 'investigational new drugs' and 'adverse drug reactions' (Dada et al., 2008).

According to our results, the service with the lowest importance ratings centered on the 'provision of information on radiopharmaceutical and chemotherapeutic drugs preparations'. This view was reinforced in a study by Karen et al. (2000) where 'provision of radiopharmaceutical drug information' was the expectation with the lowest importance rating (Sawalha, 2008). Although, there is a possible reason for this service's low importance rating (the respondent may not see a true need for the service as it relates to their practice), it would be better to ask this question in a way that health care providers who are directly involved in radiopharmaceutical and chemotherapeutic drugs preparations, could provide a separate rating for this expectation.

Using a stepwise approach, most drug information searches should begin by using tertiary literature (e.g. reference books, Micromedex Healthcare Series), followed by secondary indexing and abstracting data-bases (e.g. Medline) leading to primary literature (e.g. journal articles) when necessary (Nathan and Gim, 2009). The results of our study showed that the main drug information resources used frequently by the pharmacists for answering the patients queries are tertiary sources, such as 'drug information textbooks and medical reference books' followed by 'medical and pharmaceutical journals' and 'expert colleagues'. Similar findings were made in a study done in Singapore, assessing the need for drug and poison information, where the most used resources were 'standard text books' followed by 'expert colleagues' (Ponampalam and Anantharaman, 2003). In a paper by Rheney et al. (2000), North Carolina health care providers rarely use DPICs as sources of drug information. This was reinforced in our study, where 35.4% of the respondents almost never use this kind of drug information service. Being non acquaintance with existence of such centers and their services would be considered as the most important reason of not using them as sources of drug information. Even after 13 years of opening of the first DPIC in Iran, the health care professionals are not familiar with these centers' unique activities yet.

Expectations having a mean importance rating of ≥3 (3 = important) were reviewed to determine whether the service was currently provided. After reviewing the results, we began to develop active steps to bring the center's services closer in line with pharmacists' expectations. For expectations. For instance, as the 'provision of continuing education courses regarding drug information' had a high importance rating (3.94), we began to provide this service in our center. Pharmacists now regularly participate in our DPIC's continuing education sessions.

Conclusion

Conclusively, identifying pharmacists' expectations regarding DPIC services would be the starting point for its services improvement. At present, there is a good fit between pharmacists' expectations and DPIC services currently provided. In instances, where there was a gap between expectations and services provided, the DPIC made programmatic changes to align service with expectation when feasible.

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Full Length Research Paper

Calcium channel blockers attenuate chronic inflammation in rat knee joints

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Previous studies demonstrated therapeutic effects for calcium channel blockers (CCBs) in control of acute inflammation. There are little reports on the effect of these blockers on chronic inflammation. In this study, we investigated the effects of CCBs on chronic inflammation using complete Ferund's adjuvant (CFA) induced inflammation in the rat knee joints. This experimental study was carried out on 80 male Wistar rats. Calcium channel blockers (verapamil and nifedipine) were used in doses of 100 and 800 µg/kg. For induction of chronic inflammation, CFA (0.2 ml) was injected into the right knee joint. The changes in blood flow, and local temperature of the animal's knee joint were measured by laser Doppler flowmetry and also the knee diameter was measured by a caliper, on 0, 7, 14, 21, and 28 days after CFA injection. CFA injection significantly increased blood flow from day 1 to 28 as compared to the day 0. Administration of both verapamil and nifedipine (100 and 800 µg/kg) significantly decreased the effect of CFA on increasing blood flow (all $p < 0.05$). Administration of both verapamil and nifedipine (100 and 800 µg/kg) significantly inhibited the effect of CFA on increasing knee temperature (all $p < 0.05$). Injection of CFA increased the knee joint diameter for all 28 days after injection. Both low and high doses of nifedipine and verapamil could inhibit the increased diameter that is induced by CFA injection (all $p < 0.05$). The effects of CCBs on measured parameter were comparable with the effect of ibuprofen (which is a standard anti-inflammatory drug) on these parameters. The results revealed that CCBs inhibited the increased rat knee blood flow, diameter and temperature in chronic inflammation induced by CFA. Therefore, the underlying mechanisms for reduction of chronic inflammation possibly are modulated by these blockers.

Key words: Chronic inflammation, verapamil, nifedipine, complete Ferund's adjuvant (CFA).

INTRODUCTION

Rheumatoid arthritis is characterized as an inflammatory chronic disease with a prevalence of 1% and with various geographical distributions. Although, this disease is the focused area of research by several study groups worldwide, the new and effective therapeutic methods for rheumatoid arthritis are yet to be available (Badvi, 2000; Nkomo et al., 2010).

Vessels dilatation in parallel with increase in their blood flow is one of the complications induced by chronic inflammation, which is among the main factors in raising temperature and inflammatory edema (Khaksari et al., 2002). The joint's cartilages nutrients are supplied by synovial fluid which originated from the joint's blood flow, thus, factors that regulate the joint's blood flow play a crucial role in maintenance of joint's tissues and the homeostasis environment. Changes in the normal mechanisms in regulation of blood flow in the synovium by inflammatory diseases of joints can interfere with the

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damage of synovium (Najafipour and Niazmand, 2004). The constitutional mechanism for increasing blood flow in a chronic inflamed joint is yet to be clarified. Few reports showed that it could be due to the decreased sympathetic tonicity of the vessels (Boston et al., 2004), changes in beta-adrenergic receptors profile (Botrel et al., 1994), and the decrease in knee vessels in response to phenylephrine and overproduction of nitric oxide (NO). It has been revealed that this change is due to the altered secretion and release of local vessels relaxating mediators from the inflamed cells or sensory nerve ends, whereas calcitonin gene-related peptide (CGRP) and substance P that are released from the sensory nerves ends, caused the neurogenic dilatation of the vessels (Fahim et al., 1995). In addition, increase in the release of substance P, is possible. Local secretion of bradykinin and prostaglandins (especially prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2)) (Kaur and Halliwell, 1994), also can mediate the increase of blood flow.

Calcium ion participates in the many process, including release, activation, and effectiveness of many inflammatory mediators, such as activation of phospholipase A_2 (PLA_2), increasing the production of arachidonic acid metabolites (Chawengsub et al., 2009), NO synthesis (Koya and King, 1998), interleukins releasing, mediators of exocytosis, and chemotactic responses (Lam and Ferrell, 1993). On the other hand, evidences showed that the voltage calcium channel blockers (CCBs) cause inhibition of NO production and the enzymes responsible for the synthesis of prostaglandins (Lam and Ferrell, 1992), and decrease the release of histamine, bradykinin, serotonin, arachidonic acid metabolites, and leukotrienes (Fogel et al., 2005).

Both acute and chronic inflammatory responses lead to rise in temperature (Leventhal et al., 2005), and swelling as the two important clinical signs of inflammation, thus compounds which are used to reduce swelling and temperature is suggested to have anti-inflammatory properties. One of the leading causes of the raised temperature is the increased joint blood flow accompanied by some of the chemical mediators that are released in the site of inflammation (Fahim et al., 1995). Therefore, the factors that reduce the blood flow of joint or inhibit the production and release of mediators down regulate the joint temperature in inflammation.

Calcium play key roles in production of the factors mentioned earlier, which are involved in the inflammation, so, in a way it can be suggested that calcium blockers may be used as useful tools for treatment of inflammation. In the previous studies, the effect of voltage calcium channels on acute (Kaur and Halliwell, 1994) and chronic (McDougall et al., 1994) inflammation has been demonstrated. Therefore, the present study was designed to examined, whether if inhibitory effect of these blockers in chronic inflammation of rat knee is mediated via decreasing blood flow, temperature, and the joint diameter.

MATERIALS AND METHODS

Animals

This study was carried out on 80 adult male Wistar rats weighing 200 to 250 g. Animals were housed in Rafsanjan Faculty of Medicine animal house at a temperature of 20 to 22°C and 12 h light/dark cycle with *ad libitum* access to food and water. The following protocol was similarly carried out on all groups. All protocols were approved by the institutional animal care and use committee of Rafsanjan University of Medical Sciences.

Induction of chronic inflammation

To induce the chronic inflammation, after anesthesia by ether, 0.2 ml of complete Freund's adjuvant (CFA) was injected into the anterior space of the right knee joint by insulin syringe with 26 gage needles. This procedure is an accepted model for induction of chronic inflammations that produce rheumatoid arthritis like inflammatory reactions in human (Morgan et al., 1978; McDougall et al., 1995).

Measurement of the effects of blocker on inflamed joint

The levels of the effects of blockers on inflamed joint were measured by the following three methods.

Measurement of the changes in blood flow

To measure the changes in blood flow in response to CFA and other drugs injection, animals were anesthetized by administration of 1.5 g/kg intraperitoneally (ip) urethane. Following complete anesthesia, a two channels laser Doppler machine (DRT4 model Moor instrument, England) was used to measure the changes in blood flow (Fahim et al., 1995). The probe of this device contacted with the interior capsules of the knee and from 20 point in 1 mm square area the blood flow was recorded, and then, the average of the recorded values were assigned as the blood flow level. The changes in blood flow was measured and compared on day zero (before injection) and it continued on days, 7, 14, 21, and 28 after injection of CFA and also after oral administration of CCBs. Finally, the animals were humanely killed by the high dose of urethane. The blood flow of biological zero level (BZL) of joint's tissue were recorded to subtract from the measured level during the experiment (McDougall et al., 1995).

Measurement of the changes in temperature

Temperature of inflamed joint was another index that was evaluated in this study. Temperature was measured by the same probe in parallel with blood flow on days 0, 7, 14, 21, and 28.

Measurement of changes in joint diameter

The measurement of intralateral knee joint's diameter is a criteria to evaluate the intensity of inflammation (McDougall et al., 1995). A caliper (Diamond, China) with the precision of 0.02 mm was used to measure the right knee joint diameter on day zero (before injection) and days 7, 14, 21, and 28 after injection in different groups. The diameter before injection was compared with the diameters after injection.

Drugs and reagents

Verapamil and nifedipine (Rose Daruo Co, Iran), 100 and 800 μg/kg and ibuprofen (Sigma , UK) 15 mg/kg were administered orally by orogastric tube 7 days after injection of CFA (Biogen, Iran). These doses of drugs have shown inhibitory effect in previous studies (Najafipour and Ferrell, 1993). The drugs were dissolved in dimethyl sulfoxide (DMSO) (Merk, Germany) (Rawls et al., 2004a). The volume of oral solution was 1 ml/kg and was administered at the same time everyday from day 7 after CFA injection (onset of chronic inflammation) for 21 days forwarded. DMSO (as the solvent of drugs) was also administered orally in same volume. Urethane and ether for anesthetizing of animals were purchased from Merk (Merk, Germany).

Experimental groups

Rats were divided into 8 groups randomly and 8 to 10 animals were allocated in each group. Group I: animals that had chronic inflammation of knee by injection of CFA, and the changes in blood flow, temperature, and the joint diameter were measured for 28 days. Group II: in this group, after CFA injection, animals received DMSO (the solvent of drugs) orally from the day 7 and as group I parameters, they were measured for 28 days. Group III (control): in this group, 0.2 ml (same volume as CFA) of normal saline were injected into the right knee and the alterations were measured like group I. Group IV received verapamil (100 μg/kg) for 21 days. Group V received nifedipine (100 μg/kg), group VI received 800 μg/kg of verapamil, group VII received 800 μg/kg of nifedipine, and group VIII were injected 15 mg/kg of ibuprofen for 21 days and the change in blood flow and other two parameters were measured as in group I.

Statistical analysis

The statistical analysis was performed using excel and Statistical Package for Social Sciences (SPSS) softwares. All data are expressed as mean ± standard error of the mean (SEM). A p value of less than 0.05 has been considered as statistical significance. Repeated measurement on analysis of variance (ANOVA) was used to compare measured indices in different times. All post hoc comparisons were made using Tukey's post hoc test.

RESULTS

Changes in blood flow

The effects of CFA, saline, DMSO + CFA, and the low and high doses of verapamil and nifedipine on the knee joint blood flow in different days of study are shown in Table 1. CFA injection increased blood flow on days 7, 14, 21, and 28 as compared to the day zero (all $p < 0.01$). Despite the same blood flow on day zero in CFA, saline, and DMSO groups (99.9 ± 4.5), on the day 7 (after CFA injection), the blood flow in CFA group was significantly increased as compared to control group (229.8 ± 13.2 versus 102.1 ± 7.9, $p < 0.001$) and this was constantly maintained to the end of the study (day 28, 163.7 ± 12.4). On the other hand, DMSO did not significantly affected blood flow which was raised by CFA. Both low and high doses of verapamil and nifedipine (100 and 800 μg/kg) significantly decreased the blood flow which was raised by CFA on days 14 and 21 (Table 1) ($p < 0.001$). While on day 28, both doses of nifedipine inhibited blood flow ($p < 0.001$), but only the low dose of verapamil had a significant effect. Ibuprofen significantly inhibited the elevation of blood flow by CFA on days 14, 21, and 28 ($p < 0.001$).

Changes in knee joint temperature

The effect of CFA, saline, DMSO, and low and high doses of verapamil and nifedipine on the knee joint temperature are shown in Table 2. These results indicated that CFA injection elevated the temperature on days, 7, 14, 21, and 28 ($p < 0.001$). On day zero, not significant difference was observed between CFA, control, and DMSO groups (26.51 ± 0.58, 27.2 ± 0.4 and 27.5 ± 0.52°C respectively). Following 7 days of CFA injection, the temperature of knee joint in CFA group (35.17 ± 0.1°C) significantly increased as compared to the control (26.92 ± 0.32°C) ($p < 0.001$), and this was continued to the end of the study ($p < 0.001$). Although, DMSO had no significant inhibitory effect on temperature caused by CFA on day 14, but on days 21 (32.37 ± 0.29) and 28 (31.2 ± 0.19), the difference between DMSO and CFA group was significant ($P < 0.001$).

Low and high doses of both CCBs significantly inhibited the temperature by CFA on days 14, 21, and 28 ($p < 0.001$), there was a significant difference between low and high doses of nifedipine ($p < 0.001$) (Table 2). Indeed, on days 21 and 28, there was a significant difference between low and high doses of nifedipine and verapamil ($p < 0.001$), also on days 14 and 28, the difference in high dose of nifedipine with verapamil was significant ($p < 0.001$). Ibuprofen on days 14, 21, and 28 decreased the knee joint temperature as compared to CFA ($p < 0.001$) and there were significant differences between ibuprofen and low dose of verapamil ($p < 0.05$) and nifedipine ($p < 0.05$) on days 21 and between ibuprofen and low and high dose of nifedipine ($p < 0.01$) and verapamil ($p < 0.001$) on day 28.

The effects of different drugs on knee diameter

The comparison of the effects of different drugs on increasing the knee diameter caused by CFA injection is shown in Table 3. The knee diameter on day zero were the same as CFA, saline and DMSO groups (9.99 ± 0.08, 10.1 ± 0.07, and 9.8 ± 0.08 mm, respectively), but on days 7, 14, 21, and 28, the knee diameter in CFA group was higher than saline group ($p < 0.001$); on the other hand, DMSO did not inhibit the increased diameter caused by CFA, the knee diameter decreased on day 14 by high dose of nifedipine ($p < 0.001$) and on day 21 by low and high doses of verapamil and high dose of nifedipine ($p < 0.001$). Indeed, on day 28, the low and high doses of nifedipine and low dose of verapamil inhibited the increased diameter caused by CFA ($p < 0.01$). Ibuprofen inhibited the increased diameter caused

Table 1. Comparison of the effects of drugs on the blood flow (arbitrary) of rats' knee joint in different groups and different days of study.

Group	Day				
	0	7	14	21	28
CFA	99.9 ± 4.5	229. 8 ± 13.2	217.1 ± 11.5[b]	244.8 ± 10.4[c]	163.7 ± 11.4[d]
Saline (control)	99.8 ± 3.7	102.1 ± 7.9[a]	101.1 ± 6.1[a]	93.3 ± 1.4[a]	97. 8 ± 5.6[a]
DMSO	98.9 ± 4.6	230.5 ± 14.1	199.9 ± 13.7	232 ± 6	195.9 ± 16.9
N-100	-	240.9 ± 10.5	159.2 ± 10.9	121.3 ± 8.7	124.1 ± 6.9
V-100	-	239.4 ± 12.7	135.2 ± 21.4	112.1 ± 10.1	96.8 ± 5.1
N-800	-	230.5 ± 14.5	144.7 ± 13.7	132.7 ± 11.5	127.1 ± 8.7
V-800	-	232.8 ± 12.5	136.8 ± 14.1	148.1 ± 15	158.2 ± 10.4
Ibuprofen	-	235 ± 15.4	176.2 ± 9.5	108.1 ± 2.8	95.6 ± 5.8

Data are shown as mean SEM. CFA: complete Ferund's adjuvant, DMSO: dimethyl sulfoxide, N-100 and N-800: 100 and 800 µg/kg of nifedipine, respectively; V-100 and V-800: 100 and 800 µg/kg of verapamil, respectively; ibuprofen: 15 mg/kg. [a]Significant difference ($p < 0.001$) between saline group with CFA or DMSO group in days 7, 14, 21, and 28 of the study .[b]Significant difference ($p < 0.001$) between CFA group with different doses of verapamil, nifedipine and ibuprofen on day 14. [c]Significant difference ($p < 0.001$) between CFA group with different doses of verapamil, nifedipine, and ibuprofen on day 21. [d]Significant difference ($p < 0.001$) between CFA group with low dose of verapamil and with different doses of nifedipine and also with ibuprofen on day 28.

Table 2. Comparing the effects of drugs on the temperature (°C) of rats' knee joint in different groups and different days of the study.

Group	Day				
	0	7	14	21	28
CFA	26.51 ± 0.58[a]	35.17 ± 0.1	34.8 ± 0.35	34.16 ± 0.09	34.25 ± 0.25
Saline (control)	27.2 ± 0.4	26.92 ± 0.32[b]	26.26 ± 0.17	26.29 ± 0.29	26.71 ± 0.31
DMSO	27.5 ± 0.52	35.2 ± 0.4	33.85 ± 0.35	32.37 ± 0.29[c]	31.21 ± 0.19
N-100	-	35.3 ± 0.21	29.97 ± 0.21[d]	30.19 ± 0.26	30.25 ± 0.24
V-100	-	34.91 ± 0.35	28.38 ± 0.66[e]	28.96 ± 0.18	28.73 ± 0.14
N-800	-	35.2 ± 0.4	30.65 ± 0.39[f]	29.27 ± 0.21	29.32 ± 0.38
V-800	-	35.5 ± 0.65	28.8 ± 0.24[g]	29.63 ± 0.47	29.91 ± 0.31
Ibuprofen	-	35.5 ± 0.2	29.9 ± 0.24[h]	29.4 ± 0.12	28.1 ± 0.4

Data are shown as mean SEM. CFA: Complete Ferund's Adjuvant, DMSO: dimethyl sulfoxide, N-100 and N-800: 100 and 800 µg/kg of nifedipine, respectively, V-100 and V-800: 100 and 800 µg/kg of verapamil, respectively, ibuprofen: 15 mg/kg. [a]Significant difference ($p < 0.001$) between all days of the study as compared to day zero in CFA group. [b]Significant difference ($p < 0.001$) between CFA group with saline group on days 7, 14, 21, and 28. [c]Significant difference ($p < 0.001$) between CFA group and DMSO group on days 21 and 28. [d]Significant difference ($p < 0.001$) between CFA group and low dose of nifedipine on days 14, 21, and 28. [e]Significant difference ($p < 0.001$) between CFA group and low dose of verapamil on days 14, 21, and 28. [f]Significant difference ($p < 0.001$) between CFA group and high dose of nifedipine on days 14, 21, and 28. [g]Significant difference ($p < 0.001$) between CFA group and high dose of verapamil on days 14, 21, and 28. [h]Significant difference ($p < 0.001$) between ibuprofen and CFA group on days 14, 21, and 28.

by CFA, significantly during all the days of the study ($p < 0.01$).

On day 21, the difference between low and high doses of nifedipine was significant ($p < 0.05$). On day 14, there were significant differences between ibuprofen with low dose of nifedipine ($p < 0.01$) and low and high doses of verapamil ($p < 0.001$), whereas on day 28, there was only significant difference between ibuprofen and 800 µg/kg of verapamil ($p < 0.01$).

DISCUSSION

Previous studies reported the inhibitory role of CCBs in acute (Najafipour and Ferrell, 1993) and chronic (McDougall et al., 1994) inflammation. One of the background causes leading to pathologic events is the increased blood flow in the inflamed joints (Khaksari et al., 2002), thus, in the present study, the effects of this inhibitions in blood flow, temperature, and the diameter of inflamed joints were evaluated.

Results of the current study showed that the injection of CFA into the joint caused significant increase in the blood flow of joint from day 1, whereas on day 7 after injection of CFA, the blood flow in the injected knee increased by 121.7 ± 6.1% as compared to day zero, and then gradually decreased during the following days, but it did not reach to the initial level and it had significant difference with day zero until the end of the study (93.8 ±

Table 3. Comparison the effects of drugs on the joint diameter (mm) of rats' knee joint in different groups and different days of the study.

Group	Day				
	0	7	14	21	28
CFA	9.99 ± 0.8	13.1 ± 0.2[a]	12.4 ± 0.26	12.09 ± 0.24[e]	11.76 ± 0.18[f]
Saline (control)	10.1 ± 0.07	9.81 ± 0.08	9.71 ± 0.06	9.7 ± 0.06	9.74 ± 0.07
DMSO	9.80 ± 0.08	12.6 ± 0.08[b]	12. 3 ± 0.4	11.8 ± 0.16	11.86 ± 0.27
N-100	-	13.4 ± 0.22	11.91 ± 0.14	11.12 ± 0.29	10.68 ± 0.2
V-100	-	12. 89 ± 0.15	11.47 ± 0.16[d]	10.74 ± 0.18	10.6 ± 0.2
N-800	-	12.8 ± 0.2	11.02 ± 0.11[c]	10.07 ± 0.24	10.65 ± 0.18
V-800	-	12.9 ± 0.15	10.95 ± 0.17	10.84 ± 0.2	11.08 ± 0.19
Ibuprofen	-	13.2 ± 0.25	10.46 ± 0.27[g]	10.87 ± 0.33	9.9 ± 0.14

Data are shown as mean SEM. CFA: complete Ferund's adjuvant, DMSO: dimethyl sulfoxide, N-100 and N-800: 100 and 800 µg/kg of nifedipine, respectively, V-100 and V-800: 100 and 800 µg/kg of verapamil, respectively, ibuprofen: 15 mg/kg. [a]Significant difference ($p < 0.001$) between saline and CFA group in all days of the study. [b]Significant difference ($p < 0.01$) between DMSO and saline group on days 7, 14, 21, and 28. [c]Significant difference ($p < 0.001$) between CFA group and high dose of nifedipine on day 14. [d]Significant difference ($p < 0.001$) between CFA group and low dose of verapamil on day 14. [e]Significant difference ($p < 0.001$) between CFA group with high dose nifedipine, and also with both doses of verapamil on day 21. [f]Singnificant difference ($p < 0.001$) between CFA group with both doses of nifedipine, and also with low dose of verapamil on day 28. [g]Significant difference ($p < 0.01$) between CFA group with ibuprofen in all days of the study.

15.5% on day 28). CFA probably lead to increase in blood flow of joint with chronic inflammation by increasing the level of prostaglandins production, aggregation of phagocytes in joints and free oxygen radical production and other materials effective on vessels' diameter (Rawls et al., 2004b). The oral consumption of both calcium channel inhibitors in CFA receiving group reduced the increased blood flow of knee joint caused by CFA (the low dose of verapamil on days 14, 21, and 28, and low dose of nifedipine on days 14, 21, and 28, decreased the blood flow). The high dose of verapamil and nifedipine also reduced knee joint blood flow on the mentioned days, whereas the most inhibitory effects of verapamil (34.6 ± 6.6%) and nifedipine (42.9 ± 3.8%) was on day 14. The inhibitory effects of these blockers on this inflammation index was comparable with ibuprofen, hence, the vasodilatation caused by CFA which occurred in chronic inflammation decreases significantly by CCBs, and this indicates the role of calcium in the vessel response. CCBs possibly by altering the production and release of CGRP and substance P from the sensory neurons' terminal (Fahim et al., 1995), inhibition of PLA_2 enzyme and following that, the reduction in PGE_2 and PGI_2 production (Lam and Ferrell, 1992, Kaur and Halliwell, 1994), inhibition of calcium dependent protein kinase C (PKC) and finally the inhibition of pathway that is activated by this enzyme (Khoshbaten and Ferrell, 1990), inhibition of release or function of chemical mediators like histamine, bradykinin, serotonin and leukotrienes (Lees et al., 1998), by reduction of free oxygen radical production and superoxide (Rezaie et al., 2005) have induced their inhibition effects.

Previous studies showed that the NO level was increased during inflammatory disorders (Botrel et al., 1994; Rawls et al., 2004a). The response of smooth muscle of the vessels to the α-receptors antagonist is influenced by the released agents from endothelium of vessels (Botrel et al., 1994), whereas NO decreased vasoconstriction response in inflamed joint, thus, the CCBs may in turn causes decrease in blood flow by al-teration in the production of NO (Lam and Ferrell, 1993). It has also been reported that in acute (Rosen, 1989) and chronic (Morgan et al., 1978) inflammation in rat knee, the constriction response to the knee sympathic stimuli-tion has decreased, so probably CCBs, prevented the decrease in responsiveness by local changes in the inflamed joint.

The results of other part of this research indicated that the low dose of verapamil and nifedipine had the most inhibitory effects on the reduction of the inflamed joint temperature that was 16.7 ± 1.2 and 12.05 ± 1.2%, respectively. On day 14, with the high dose of nifedipine and verapamil, the greatest effect was observed on day 21, 16.1 ± 1.5 and 15.2 ± 1.7%, respectively. The CCBs possibly via reduction of NO (Mustafa and Olson, 1999) inhibited the release of neuromediators as amma-aminobutyric acid (GABA) (Shirota et al., 1988), reduction of interleukins release (Sobal et al., 2001), prostaglandins (Arend and Dayer, 1995), or lessened the blood flow that was observed in the present study that caused the decrease in the joint temperature. The useful inhibiting effect of CCBs on reduction of temperature in this study is consistent with the reports of the useful effect of these drugs on decreasing the temperature in ovarectomized animals (Wirth et al., 1992), Reynald syndrome (Zeni and Ingegnoli, 2004), and inflamed paw (Arend and Dayer, 1995).

The results of the current study also indicated that CFA injection in the knee joint caused significant increase of knee diameter on day 3 and it reached to the maximum and from day 7 the increase was constant up to day 28

(end of study). These results are consistent with McDougall et al. (1995) who reported that, after knee joint CFA injection, the chronic inflammation occurred on day 7 (McDougall et al., 1995). CFA possibly run a chronic inflammation or arthritis like phenomenon via increasing of PGE_2 production, reduction of sulfhydryl (SH) group in serum, increase in blood glutathione (GSH) production, phagocytes aggregation in joint, and free oxygen radical and super oxide production (Rawls et al., 2004b).

Treatment of chronically inflamed animals with both CCBs reduced the knee joint diameter, and the most inhibitory effects of low dose of verapamil on day 21 was 11.2%, while its high dose was on days 14 was 11.38% and 21 was 10.41%, respectively. Also, the increase in knee joint diameter (8.7%) caused by CFA, inhibited low dose of nifedipine on day 28 and by high dose of this drug (11.6%) on day 21 as compared to day zero. The inhibitory effect of these CCBs was comparable to the effect of ibuprofen, especially on day 21, although, the inhibitory effect of ibuprofen on day 28 was greater than both doses of CCBs.

Overall, findings of the present study indicated that both verapamil and nifedipine exhibited considerable and powerful effect on the reduction of blood flow, temperature, and knee joint diameter in chronic inflammation. The anti-inflammatory effects possibly occurred via reduction in blood flow, decrease in the temperature and reduction of the diameter. This study also showed that calcium ion plays a role in the processes of rheumatoid arthritis, indicating that CCBs could probably be introduced as new therapeutic targets which are effective in this chronic disorder, and to confirm this claim, clinical trial studies should be done.

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Full Length Research Paper

Osteoblastic activity of ethanolic extract and volatile compounds from Ee-Zhi-Wan, a famous traditional Chinese herbal formula

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Er-Zhi-Wan (EZW), a famous traditional Chinese formula, which is comprised of the aerial parts of *Eclipta prostrate* L. (EP) (Astraceae) and the fruits of *Ligustrum lucidum* Ait. (FLL)(Oleaceae) has been developed as a restorative formula for hundreds of years. It is widely used to prevent and treat various kidney diseases. This study aimed to investigate the effects of volatile components and ethanolic extract from EZW on the proliferation and differentiation of primary osteoblasts by the MTT method and measuring the activity of alkaline phosphatase (ALP). Both ethanolic extract and volatile components from EZW could significantly (p<0.01) stimulate the proliferation and increase the ALP activity of primary osteoblasts. The volatile components of EZW were analyzed by GC-MS. A total of 61 compounds, which were the major part (about 86.34%) of the volatiles were identified by matching mass spectra with a mass spectrum library (NIST 05.L) and retention indexes (RI) of the compounds reported on equivalent column.

Key words: Er-Zhi-Wan (EZW), volatile compounds, osteoblasts, GC-MS.

INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by low bone mass and micro-architectural deterioration of bone tissues, resulting in increased bone fragility and susceptibility to fracture (Rodan and Martin, 2000). Post-menopausal osteoporosis is a major health problem with significant morbidity and mortality (Cummings et al., 1990). Classical hormone replacement therapy (HRT) has been recommended to prevent and treat post-menopausal osteoporosis for many years. However, in 2002, the American National Institute of Health stopped a clinical trial with HRT in healthy post-menopausal women due to the higher incidence of breast cancer, heart attack, and stroke and blood clots (Rossouw et al., 2002). Traditional Chinese medicines have been applied to prevent and treat post-menopausal osteoporosis in clinical practice for thousands of years, and these medicines with fewer side effects are more suitable for long-term use compared with chemically synthesized medicines. In addition, according to the law of compatibility of traditional Chinese medicines, a single herbal medicine usually exerts a limited therapeutic action. When several herbal medicines are mixed in a certain proportion, they

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will display their superiority over a single herb in the treatment of a disease (Qin et al., 2008). As such, traditional Chinese formulas will undoubtedly be a cost-effective alternative to commercial pharmaceutical products. Er-Zhi-Wan (EZW), a famous traditional Chinese formula firstly recorded in “Yi Bian” written in Ming Dynasty, is comprised of the aerial parts of *Eclipta prostrata* L. *(EP) (Astraceae) and the fruits of Ligustrum lucidum* Ait. (FLL) (Oleaceae). It possesses the actions of tonifying the liver and kidney, nourishing the body’s essential fluid, and arresting hemorrhages (Chinese Pharmacopoeia Committee, 2005). According to the traditional Chinese medicine theory, “kidney” controls bone. The “kidney-tonifying” action of traditional Chinese medicine might have relationship with bone formation (Wang et al., 2009; Zhang et al., 2008). Moreover, our previous studies have shown that both ethanolic extract and volatile compounds from EP and FLL could significantly stimulate the proliferation and increase the ALP activity of rat calvarial osteoblasts (Lin et al., 2010; Wu et al., 2011). We also found that the serum from rats treated with aqueous extract of EZW did not facilitate proliferation of rat calvarial osteoblasts and UMR106 cells, but evidently inhibited both proliferation of RAW264.7 cells and differentiation of osteoclasts from RAW264.7 cells induced by receptor activator of nuclear factor $_{K}$B ligand (RANK-L) and macrophage-colony stimulating factor (M-CSF) (Zhang et al., 2008). However, the effects of ethanolic extract and volatile compounds from EZW on the proliferation and differentiation of rat calvarial osteoblasts, and whether it has better effects than EP or FLL, still have not been investigated.

Many researches showed that volatile compounds obtained from many plants are responsible for their pharmacological activities just as non-volatile compounds in herbs (Lograda et al., 2010; Ho et al., 2010; Wei et al., 2012; Sharma et al., 2012; Rahimi et al., 2011). Both EP and FLL contain rich volatile compounds, from which we could always smell the strong fragrance. However, as far as our literature survey could ascertain, there is no report on any investigation on the volatile compounds from EZW.

According to the aforementioned knowledge and researches in the present study, we investigated the effects of ethanolic extract and volatile compounds from EZW on the proliferation and differentiation of rat calvarial osteoblasts.

MATERIALS AND METHODS

Plant material

The aerial parts of *Eclipta prostrasta* L. (20080710) and the fruits of *L. lucidum* Ait. (Oleaceae) (20080601) were purchased from Fujian Tianren Pharmaceutical Company and identified by Professor Cheng-zi Yang of the Department of Pharmacy, Fujian University of Traditional Chinese Medicine. The voucher specimens of these fruits were deposited at the Herbarium of Department of Pharmacognosy, Fujian University of Traditional Chinese Medicine, Fuzhou, P. R. China.

Chemicals and reagents

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl suphoxide (DMSO) were purchased from Sigma (U.S.A.), and n-Alkanes C_8 to C_{40} were purchased from Accustandard (U.S.A.). Phenol red-free Dulbecco’s modified Eagle’medium (phenol red-free DMEM) and fetal bovine serum (FBS) was purchased from Hyclone (U.S.A.). Ethanol, diethyl ether, anhydrous sodium sulphate, diethanolamine, disodium-4-nitrophenyl phosphate, and 4-nitrophenol were of domestic AR grade. Luteolin and ursolic acid standards were purchased from the National Institute for the Control of Pharmaceutical and Biological Products with a purity of >98%, and n-Alkanes C_8-C_{40} were purchased from Accustandard (U.S.A.). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

Ethanolic extracts and volatile compounds preparation

EZW was extracted by the method described previously by Lin et al. (2010), the same prepared procedures with EP and FLL. Briefly, the powder of EZW 10g (EP: FLL = 1: 1, w/w) mixed with 100 ml of 75% (v/v) aqueous ethanol was loaded into a flask equipped with a water condenser tube. The extraction solvent was boiled (80 ± 2°C) and refluxed for a period of 120 min. Extraction was repeated twice. The combined extracts were filtered through filter paper and evaporated to dryness in a rotary evaporator (RE-52, Shanghai splendor and biochemical instrument Co., China) at 45°C under reduced pressure to yield the crude ethanolic extract. The dry material of EZW 300 g (EP: FLL= 1: 1, w/w) was crushed (40 mesh), then soaked in 3000 ml water for about 12 h before they were subjected to hydro-distillation in a Clevenger type apparatus. The contents were distilled for 3 h to obtain the volatile oils with yellowish colour and a pleasant smell and the oils were then dried over anhydrous sodium sulphate. The experiment was repeated thrice. The mean recovery of volatile oils was 0.37 ± 0.032% (w/w). The oils were stored at 4°C in the dark until tested and analyzed.

Preparation of test samples

Both ethanolic extract and volatile compounds from EZW were dissolved in dimethylsulfoxide (DMSO) at concentration of 10 mg/ml, and diluted in culture medium to the working solution before use. To avoid DMSO toxicity, the concentration of the solvent was less than 1% (v/v). For effects of steroids on growth or differentiation, culture media was charcoal stripped and without phenol red.

Cell cultures

Sprague–Dawley rats, which were 2 to 3 days old, were purchased from the Experimental Animal Center of the Fujian Medical University, Fuzhou, P.R. China. Primary osteoblastic cells were prepared from the calvarias of newborn rats following the sequential enzymatic digestion method (Idris et al., 2008). Briefly, skull (frontal and parietal bones) were dissected; then the endosteum and periosteum were stripped off, and the bone was cut into approximately 1 to 2 mm^2 pieces and digested sequentially using trypsin (0.25%, w/v) for 30 min and collagenase II (1.0 mg/ml) containing 0.05% trypsin (w/v) for 2 h. The cells were collected and cultured in phenol red free DMEM supplemented with 10% FBS and 1% penicillin/streptomycin for 24 h in a humidified atmosphere of 5% CO_2 in air at 37°C and then, the media was changed.

Assay for osteoblast proliferation and ALP activity

Primary osteoblasts (2×10^4 cells/well) were subcultured into 96-well culture plates, and incubated 24 h before the addition of test samples or control (DMSO, final concentration was 1% v/v.), and cultured again for another 48 h. Prior to the end of culture, MTT (20 μL and 5 mg/ml) was added to each well and incubated for 4 h, after which the medium was discarded, and 150 μL of DMSO was added to each well. The cells were incubated for 20 min. The UV absorbance was measured at 490 nm at a microplate spectrophotometer (Bio-rad Model 680, USA) with a reference at 630 nm and used as an indicator of osteoblast proliferation. Proliferation (%) was calculated as 100 × (OD of volatile compounds - treated / OD of control), where OD is the average absorbance of six experiments with 8 replicates. Primary osteoblasts were seeded at 2×10^4 cells/well in 96-well culture plates, and treated with test samples or control for 9 days (Media was changed per three days). The ALP activity was measured according to the literature (Owen, 1990). Total protein was assayed by the method of Bradford (Bradford, 1976). The ALP activity was expressed as micromoles of 4-nitrophenol liberated per milligram protein.

Quantification of the total triterpene acids (TTA) by the colorimetric method

After optimizing all experiment parameters, the content of the TTA was determined by the colorimetric method (Fan et al., 2006) with the following procedure. The suitable amount of each ethanolic extract was obtained as described in ethanolic extracts and volatile compounds preparation was dissolved in 50 ml of ethanol, respectively. Ethanol solution (0.2 ml) was added to the graduated test tube and evaporated to dry in a boiled water bath, and then 0.3 ml of 5% vanillin/glacial acetic acid (w/v) and 1 ml of perchloric acid solution were added to the tube successively. The sample solution was heated for 20 min at 70°C and then cooled in an ice–water bath to the ambient temperature. The absorbance of the sample was measured at 550 nm using an ultraviolet–visible spectrophotometer (Shanghai Laipade Science Instruments Co., Ltd.) after 5 ml of glacial acetic acid was added. Ursolic acid was then used as the standard.

GC–MS analysis

GC–MS analysis was performed on an Agilent 6890N Network GC System, fitted with a HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; maximum temperature, 350°C), coupled to an Agilent 5975 inert XL Mass Selective Detector. Ultrahigh purity helium (99.999%) was used as carrier gas at a constant flow of 1.0 ml/min. The injection, transfer line and ion source temperatures were 250, 250 and 200°C, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 35 to 500 amu. The splitless injection was employed for the analysis. The diluted sample (10 mg/ml, in redistilled diethyl ether) volume injected with an Agilent 7683B series injector was 1 μL. The oven temperature program was 90 to 2.5°C /min to 130 to 1.2°C /min to 170 to 2°C /min to 230 to 2°C / min to 250°C (5 min).

Identification and quantification of volatile compounds

Volatile compounds were first identified by comparing the spectra obtained with a mass spectrum library (NIST 05.L). Corroboration of the identification was then sought by matching the mass spectra of compounds with those present in the literatures and the retention indexes of the compounds reported on equivalent column (Cardile et al., 2010; Ogunbinu et al., 2009). Compounds relative percentages were calculated from the TIC from the automated integrator.

Statistical analysis

All data were presented as mean values of three determinations ± S.D. The results of all mean values were analyzed by one-way ANOVA and Turkey-HSD at $p < 0.01$ to detect significant differences among groups.

RESULTS

Proliferation and alkaline phosphatase activity assays

As the tested samples with different concentrations were added to wells for 48 h, both of which (1 μg/ml to 100 μg/ml) dose-dependently (p<0.01) stimulated the proliferation of rat calvarial osteoblasts, except ethanolic extract 1 μg/mL (Figure 1). The maximal effect was observed when cells were incubated with ethanolic extract 100 μg/ml. To ascertain whether EZW is capable of affecting osteoblastic cell differentiation, we examined the changes in ALP activity. As shown in Figure 2, both ethanolic extract and volatile compounds significantly (p<0.01) increased ALP activity in osteoblasts over the 9 days, and the maximal effects of them were observed when cells were incubated with 10 μg/ml and 1 μg/ml, respectively. Therefore, EZW could stimulate osteoblastic activity at least in part by enhancing synthesis of ALP.

Validation of the colorimetric method and the content of total triterpene acids (TTA) in EZW

Under the optimal colorimetric method condition used in this study, calibration curve was constructed in the range 0.02 to 0.08 mg. The regression equation of the curve was calculated as follows: $y = 7.3786x - 0.0091$ (y is the absorbance of the sample solution after chromogenic reaction and x is the weight of ursolic acid or TTA (mg), the correlation coefficient of the regression equation (r^2) was 0.9992. The precision and repeatability of this method were evaluated by replicate (n = 5) analysis of the same sample and by analysis of five independently prepared samples. Analysis of a sample after standing at room temperature for 0, 30, 60, 90 and 120 min was also conducted to assess sample stability. Both the RSD (%) of precision and repeatability were less than 1.0%. The sample was stable during 120 min (RSD% = 0.72%). The recovery assay of the TTA was carried out by adding the standard to the treated materials, and the recovery was in the range of 95.79 to 99.61%. The amount of TTA in raw materials of EZW, FLL and EP were 2.00 ± 0.011%, 3.98 ± 0.037%, 0.51 ± 0.006%, respectively.

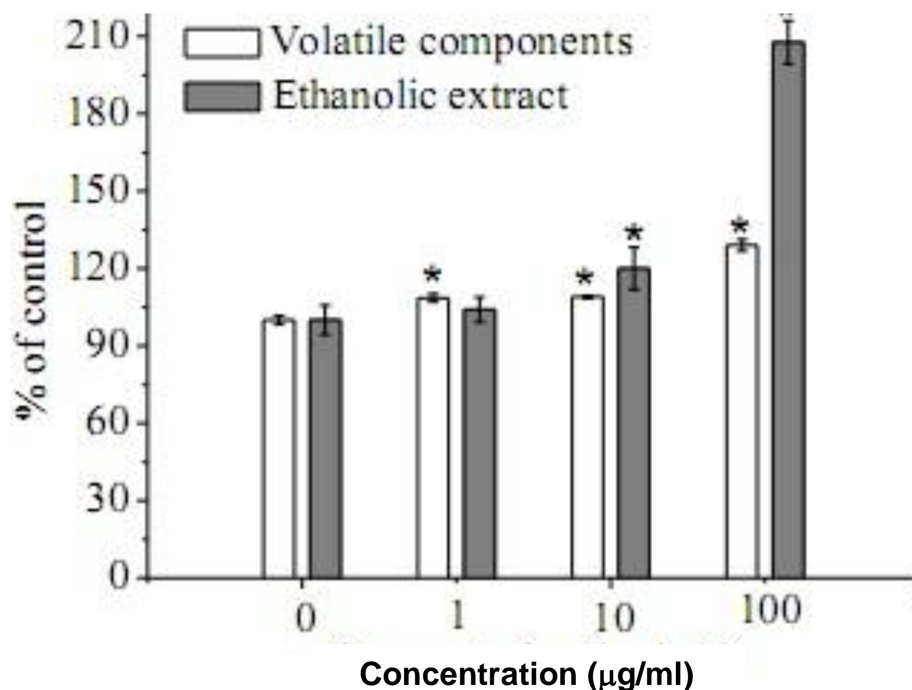

Figure 1. Effect of ethanolic extract and volatile components from EZW on the proliferation of rat calvarial osteoblasts (n=8, $\bar{x}$ ±SD; * p<0.01, compared with control).

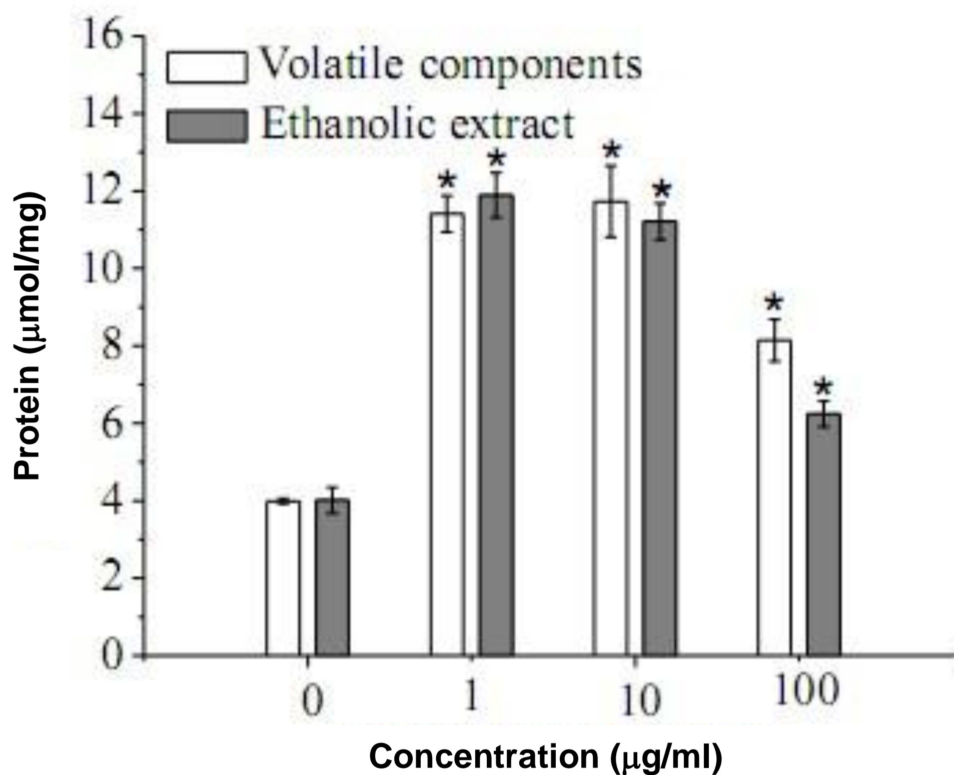

Figure 2. Effect of ethanolic extract and volatile components from EZW on the ALP activity of rat calvarial osteoblasts (n = 8 and $\bar{x}$ ± SD; * p<0.01, compared with control).

Table 1. Volatile components from EZW.

Peak no.	Component	RT (min)	RI[a]	Peak area (%)
1	1-Methyl-4-(1-methylethyl)-benzene	1027	1027	0.02
2	D-Limonene	1032	1032	0.10
3	4-Methyl-1-(1-methylethyl)-3-cyclohexen-1-ol	1181	1181	0.03
4	(S)-α, α, 4-Trimethyl-3-cyclohexene-1-methanol	1195	1195	0.03
5	6,6-Dimethyl-bicyclo[3.1.1]hept-2-ene-2-methanol	1202	1202	0.07
6	2-Methyl-3-phenyl-propanal	1241	1241	0.04
7	n-Decanoic acid	1365	1365	0.07
8	α-Cubebene	1377	1377	0.04
9	1,2-Dimethoxy-4-(2-propenyl)-benzene	1405	1405	0.05
10	[1R-(1R*,4Z,9S*)]-4,11,11-Trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene	1408	1408	0.09
11	Caryophyllene	1419	1419	0.07
12	Z,Z,Z-1,5,9,9-Tetramethyl-1,4,7,-cycloundecatriene	1451	1451	0.48
13	(Z)- 7,11-Dimethyl-3-methylene-1,6,10-dodecatriene	1454	1454	0.10
14	(4aR-trans)- Decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-naphthalene	1473	1473	0.11
15	(1α,4aα, 8aα)- 1,2,3,4,4a,5,6,8a-Octahydro- 7-methyl-4-methylene-1-(1-methylethyl)-naphthalene	1474	1474	0.09
16	[s-(E,E)]- 1-Methyl-5-met hylene-8-(1-methylethyl)-1,6-cyclodecadiene	1479	1479	0.14
17	Pentadecane	1500	1500	2.15
18	(S)-1-Methyl-4-(5-methyl-1-methylene-4-hexenyl)-cyclohexene	1507	1507	0.09
19	1,2,4a,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	1510	1510	0.24
20	(1S-cis)- 1,2,3,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	1518	1518	1.29
21	[1R-(1α,4aα,8aα)]- 1,2,4a,5,6,8a-Hexahydro-4,7-dimethyl-1-(1-methylethyl)-naphthalene	1528	1528	0.10
22	[S-(Z)]-3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol	1551	1551	0.15
23	Dodecanoic acid	1556	1556	0.49
24	[1aγ-(1aα,4aα,7β,7aβ,7bα)]-Decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulen-7-ol	1561	1561	0.19
25	Caryophyllene oxide	1565	1565	0.87
26	1-(4-Ethylphenyl)-3-methyl-pyrazol-(4H)-one	1570	1570	0.27
27	Cedrol	1580	1580	0.20
28	Hexadecane	1584	1584	0.32
29	1,2,3,4,4a,7-Hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	1607	1607	0.78
30	α-Cadinol	1628	1628	4.85
31	[1S-(1α,4α,4aβ,8aβ)]-1,2,3,4,4a,7,8,8a-Octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol	1629	1629	1.00
32	[1S-(1α,7α,8aα)]- 1,2,3,5,6,7,8,8a-Octahydro-1,8a-dimethyl-7-(1-methylethenyl)-naphthalene	1632	1632	0.18
33	[1aR-(1aα,4aβ,7α,7aβ,7bα)]-Decahydro-1,1,7-trimethyl-4-methylene-1H-cycloprop[e]azulene	1657	1657	0.16
34	Z-1,6-Tridecadiene	1660	1660	0.50
35	8-Heptadecene	1669	1669	0.55
36	Heptadecane	1700	1700	4.59

Table 1. Contd.

37	2-Pentadecanol	1704	1704	0.12
38	Hexadecanal	1713	1713	0.52
39	3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	1719	1719	0.26
40	Phenanthrene	1753	1753	0.12
41	Tetradecanoic acid	1768	1768	1.12
42	6,10,14-Trimethyl-2-pentadecanone	1844	1844	3.20
43	2- Chloropropionic acid, octadecylester	1847	1847	0.30
44	Pentadecanoic acid	1867	1867	0.63
45	6,10, 14-Trimethyl-5,9,13-pentadecatrien-2-one	1914	1914	0.21
46	3-Methyl-2-(3,7,11-trimethyldodecyl) furan	1916	1916	0.10
47	Hexadecanoic acid methyl ester	1927	1927	0.76
48	9-Hexadecenoic acid	1943	1943	0.19
49	n-Hexadecanoic acid	1988	1988	20.58
50	9,12-Octadecadienoic acid methylester	2092	2092	1.38
51	(E)-8-Octadecenoic acid, methylester	2099	2099	1.82
52	Octadecanoic acid methyl ester	2128	2128	0.08
53	(Z,Z)-9,12-Octadecadienoic acid	2156	2156	23.11
54	(E)-9-Octadecenoic acid	2165	2165	8.89
55	2,2':5',2''-Terthiophene	2177	2177	0.29
56	Tricosane	2300	2300	0.22
57	Tetracosane	2400	2400	0.05
58	2,2'-Methylenebis[6-(1,1-dmethylethyl)-4-methyl-phenol	2408	2408	0.11
59	Pentacosane	2500	2500	0.27
60	1,2-benzenedicarboxylic acid diisooctyl ester	2544	2544	1.44
61	Heptacosane	2700	2700	0.07
	Total			86.34

[a] RI: Retention indexes relative to n-alkanes C_8-C_{40} on HP-5MS column.

Volatile compounds analysis

All of 61 compounds, which were the major part (86.34%) of the volatiles, were identified. GC–MS profile of the volatile compounds showed the presence of a wide range of compounds, including terpenoids, aromatics, long-chain hydrocarbons, alcohols, aldehydes, ketones, acids and esters. The retention indexes and percentage composition are given in Table 1, where the compounds were listed in order of elution from a HP-5MS column. The main compounds were as follows: (Z,Z)-9,12-octadecadienoic acid (23.11 ± 1.099%), n-hexadecanoic acid (20.58 ± 1.156%), (E)-9-octadecenoic acid (8.89 ± 0.579%), α-cadinol (4.85 ± 0.263%), heptadecane (4.59 ± 0.393%), 6,10,14-trimethyl-2-pentadecanone (3.20 ± 0.262%), pentadecane (2.15 ± 0.033%), (E)-8-octadecenoic acid methyl ester (1.82 ± 0.136%), 1,2-benzenedicarboxylic acid diisooctyl ester (1.44 ± 0.145%), 9,12-octadecadienoic acid methyl ester (1.38 ± 0.145%), (1S-cis)-1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1 methylethyl)-naphthalene(1.29±0.146%), tetradecanoic acid (1.12 ± 0.032%), and [1S-(1α,4α,4aβ,8aβ)]-1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol (1.00 ±

0.016%).

DISCUSSION

The theory of traditional Chinese medicine believes that bones are governed and dominated by the “kidney”, which means that the “kidney” plays an important role in growth and formation of bones. Strong “kidney” can nourish bone and makes it flourish, but the weak “kidney” makes bone perish (Luo et al., 2006). In addition, according to the law of compatibility of traditional Chinese medicines, compound recipe often displays its superiority over a single drug in the treatment of a disease (Qin et al., 2008). EZW, a famous “kidney-tonifying” traditional Chinese medicine formula, which is widely used to prevent and treat various kidney diseases for its actions of nourishing the kidney, might be beneficial to bone formation. In EZW formula, there are various kinds of chemical constituents. The flavonoids from FLL and EP possess the estrogen-like activity (Lin et al., 2009). These kinds of compounds, which have aroused general concern, have the capacity to bind to the estrogen receptors and maybe decrease the bone loss like estrogen (Messina et al., 2000). This further verified the thoughts of traditional Chinese medicine that the bone could be strengthened by nourishing kidney.

Many studies have demonstrated that FLL could significantly improve the total, cortical and trabecular bone mineral density in lumbar spine and promote osteogenesis and suppress adipogenesis in MSCs as indicated by the elevated alkaline phosphatase activity, calcium deposition levels and decrease adipocyte number without cytotoxic effects (Ko et al., 2010). Moreover, FLL extract could inhibit high bone turnover, elevate intestinal calcium absorption and prevent calcium loss in young ovariectomized rats (Zhang et al., 2006). In addition, the methanol extract of aerial parts of EP was found to increase the ALP activity significantly in primary cultures of mouse calvarial osteoblasts (Lee et al., 2008). These findings give some insight into the antiosteoporotic mechanism of EZW.

In the present study, we investigated the effects of ethanolic extract and volatile compounds from EZW on the proliferation and ALP activity of rat calvarial osteoblasts. Our results showed that both ethanolic extract and volatile compounds could significantly (p<0.01) stimulate osteoblast proliferation in a dose-dependent manner and increased the ALP activity. By comparing the effects of ethanolic extracts and volatile compounds from EZW and every single herb (EP or FLL) we have studied (Lin et al., 2010; Wu et al., 2011), we found that the volatile compounds from EZW (compatibility of two herbs) displayed the similar effects on proliferation and ALP activity with those from every single herb. The ethanolic extract from EZW was also not superior to the ethanolic extract from every single herb on the enhancement of ALP activity. However, the ethanolic extract from EZW possessed a stronger osteoblastic proliferative activity than ethanolic extract from every single herb. This result displayed the advantage of compatibility of traditional Chinese medicine formula.

Total triterpene acids (TTA) such as ursolic acid might be main active compounds in EZW. Previous publication reported that ursolic acid could enhance differentiation and mineralization of osteoblasts in vitro and stimulate bone-forming in vivo in a mouse calvarial bone formation model (Lee et al., 2008). In addition, some other titerpenoids were also shown to have anti-osteoporotic activity (Li et al., 2007). Therefore, quantification of the TTA is necessary for the quality control of EZW. Moreover, both EP and FLL contain rich volatile compounds, the volatile compounds analysis can be considered as a complementary measure of quality control of EZW.

Conclusion

EZW with the potential to stimulate osteoblast proliferation and differentiation might be used as an alternative therapeutic agent for prevention and treatment of osteoporosis and display its superiority over a single herb (EP or FLL). Further studies on the isolation of anti-osteporotic fractions and constituents in EZW are in progress.

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Full Length Research Paper

Effect of cultivation condition on the active component contents in *Gentiana rigescens*, a traditional Chinese medicine

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The study aimed to determine the contents of secoiridoids activity compounds in different organs of *Gentiana rigescens,* and investigate the effects of growing age and cultivation method on the secoiridoids contents. The active compound contents in the root, the major medicinal part of *G. rigescens,* were higher than those in other organs. The active compounds contents in the leaf and flower were the highest among aerial parts. Effect of growing age and cultivation method on quality of the root was less significant than the aerial parts. The *G. rigescens* grown for 2 years could reach the requirement of the active contents in China.

Key words: *Gentiana rigescens,* chemical content, gentiopicroside, swertiamarin, sweroside.

INTRODUCTION

Traditional Chinese medicine (TCM) has a long history in Asian health system and attracted considerable attention in Europe and America (Drăsar and Moravcova, 2004; Elena et al., 2010; Grayson, 2011). Medicinal plant has long been the principal tools of TCM and development in the country's medical treatment system (Lambert et al., 1997; Capasso, 2003). In the past, wild medicinal plants were the main source of herbs in the market. As there is an increase in the demand of the medicinal industry's material, cultivation would the main hope for sustainable supplies (Lambert et al., 1997).

Recently, for the increasing effort of TCM study, a large number of bioactive compounds have been isolated and studied from medicinal plants (Calvin, 2011; Olorunnisola et al., 2012; Khalil et al., 2012). Those bioactive compounds, such as artemisinin (Qinghaosu), were the key effect of TCM (Klayman, 1985; Normile, 2003; Tu, 2011). Most bioactive compounds in medicinal plants were secondary metabolites. The accumulation of those compounds influence the quality of TCM (Su et al., 2005).

Secondary metabolite content was usually affected by environmental changes. They play an important role in adaptation of plant to the variation of habitat and overcoming stress constrains (Edreva et al., 2008). *Gentiana rigescens* is a member of the family Gentianaceae. It is a kind of endemic plant in China and is one of the oldest documented traditional Chinese herbs. *Dian Nan Ben Cao*, a book about medicinal plants of Yunnan in China, first mentioned the use of the root of *G. rigescens* for liver ailments, sore throat and eye disease in 1436. In recent years, *G. rigescens* extract was reported to have some other biological activities, such as antifungal and neuritogenic activities (Xu et al., 2007, 2009; Gao et al., 2010). Currently, *G. rigescens* root is mainly used for the effective treatment of rheumatoid arthritis, hepatitis and cholecystitis. It has been one of the best selling herbal products in China (Liu et al., 2000; Li et al., 2009). Secoiridoids, belonging to terpene, were the major pharmacological active constituents and secondary metabolites of *G. rigescens*. Gentiopicroside, swertiamarin and

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Table 1. Sample collection.

Cultivation method	Growing age	Sample number	Collection organs
Type I	2	10	Root, stem, leaf, flower
	3	10	Root, stem, leaf, flower
Type II	2	10	Root, stem, leaf, flower
	3	10	Root, stem, leaf, flower
Type III	2	10	Root, stem, leaf, flower
	3	10	Root, stem, leaf, flower

sweroside as secoiridoid component were believed to contribute to the therapeutic functions of medicinal materials of *G. rigescens* was evaluated by *G. rigescens* (Shen et al., 2010). Traditionally, the quality mouth feel and appearance. Now, for the revelation of bioactivities research, contents of bioactive compounds (especially gentiopicroside) were the most important evaluation index (Committee for the Pharmacopoeia of P.R. China, 2010).

In the past, wild medicinal plants were the major source of medicinal materials. However, as the quantity demand of medicinal materials increased rapidly, more and more cultivated medicinal plants like *G. rigescens*, became the new sources of TCM. Cultivation condition would influence the accumulation of active compounds and the quality of medicinal plant (Chen et al., 2010). Different cultivation methods usually provide a change of habitat, such as light, soil and shading conditions (Zhao et al., 2005; Cao et al., 2005; Wang et al., 2006; Huang et al., 2009; Du et al., 2009). Studies on influence of cultivation condition on the accumulation of active compound in *G. rigescens* were quite limited. In the current work, we compared three major active constituents content of *G. rigescens* under different cultivation conditions from Lincang of Yunnan in China. The aim was to provide useful information for the quality control and cultivation technique improving of *G. rigescens* in Yunnan.

MATERIALS AND METHODS

Plant materials

Plant material was collected from Lincang, a major *G. rigescens* producing area in China, in November, which corresponded to the traditional harvest period. Sample came from three types of cultivation method: i) planting *G. rigescens* on slope of the valley without shading, ii) planting herbs in the tea garden shading with young tea (2 to 2.5 years old), and iii) culturing under adult tea (10 years old). All samples were divided into different parts (root, stem, leaf and flower) (Table 1).

Preparation of samples

The samples were dried in a temperature of 50°C and ground to fine powder using a pestle mortar.

Extraction

Two hundred and fifty milligram of the fine powder was taken from each sample and extracted in 5 ml of 99.9% methanol for 40 min in an ultrasound cleaner. 1.5 ml of the extract was taken in a 2 ml centrifuge tube and centrifuged at16,000 rpm for 10 min; after which, the extracts were cooled to room temperature.

Chemicals and reagents

The standard gentiopicroside, swertiamarin, and sweroside were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was purchased from TEDIA (Ohio, USA). HPLC grade water was purchased from Milli-Q system (Massachusetts, USA). Other reagents were of analytical grade.

Instrumentation and analytical conditions

The HPLC system (Shimadzu Technologies, Kyoto, Japan) was equipped with the Workstation software class-VP (Shimadzu Technologies) and composed of HPLC-10 Integrator, HPLC-10ATVP pump, SPD-M10A VP detector (DAD). All chromatographic separations were carried out on a Shim-pack VP-ODS C18 (150 ×4.6 mm, particle size: 5 μm) from Shimadzu (Kyoto, Japan). Mobile phase was methanol: water (30: 70). The flow-rate was 1.0 ml/min, injection volume: 10 μl, column temperature: 25°C. Detection wavelength were; gentiopicroside under 270 nm, swertiamarin and sweroside under 240 nm. The peak identification was based on the retention time and the DAD spectrum against the standard presented in the chromatogram.

Preparation of standard solutions

Standard stock solutions were prepared by directly dissolving the standard substances of the three compounds in methanol, respect-tively, prepared for HPLC analysis and establishment of calibration curves. All standard stock solutions for HPLC were filtered using 0.25 μm syringe filter before injection.

Statistical analyses

PASW Statistics (version 18.0) was used for data processing and analyses. Data are mean values ± SD for three replicates. The means of the treatments were separated using T-test and one-way

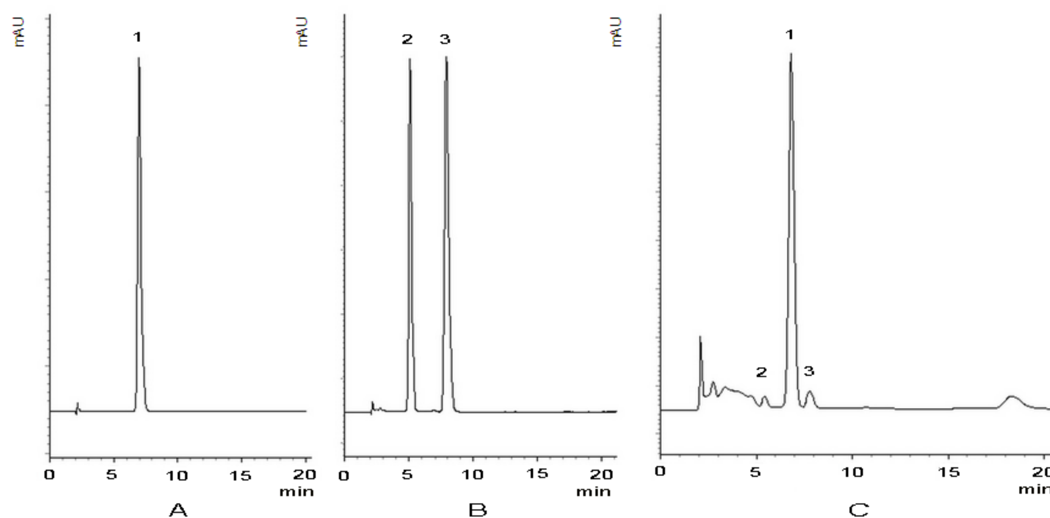

Figure 1. HPLC chromatogram showing presence of reference substance and sample, (A) gentiopicroside, (B) swertiamarin (2) and sweroside (3), (C) HPLC chromatogram of active components extracted from *G. rigescens*.

Table 2. Calibrations of gentiopicroside, swertiamarin, and sweroside.

Standard	Regression equation	Concentration of standard (mg/ml)	*R*	Recovery (%)
Gentiopicroside	Y= 1986934.5X - 495519.9	0.040-0.80	0.9997	100.04
Swertiamarin	Y= 1385510X - 579027.2	0.002-0.040	0.9996	98.48
Sweroside	Y = 1819411.286X - 1135506.686	0.002-0.040	0.9987	98.46

ANOVA analysis at a 0.05 significance level.

RESULTS

Qualitative analysis

Standard of gentiopicroside, swertiamarin and sweroside were run on HPLC, respectively. The chromatograms and calibrations are shown in Figure 1 and Table 2. The single peaks of gentiopicroside, swertiamarin and sweroside were obtained at the retention time of 6.9, 5.4 and 7.9 min, respectively.

Active compounds content in different organs of *G. rigescens.*

Contents of gentiopicroside, swertiamarin and sweroside in the different organs of *G. rigescens* are presented in Figures 2 to 4. The three kinds of active compounds could be found in roots, stems, leaves and flowers. Content of gentiopicroside was the highest among active compounds in every organ.

Gentiopicroside are mainly distributed in root (38.005 and 35.526 mg/g), while sweroside are mainly distributed in flower (0.862 and 0.975 mg/g). The content of swertiamarin in root and stem were higher than other organs.

Effect of various cultivations of gentiopicroside content

The variation of gentiopicroside content of *G. rigescens* is shown in Table 3. In the same cultivation method, the content of gentiopicroside in most samples were mildly influenced by growing age (P>0.05). However, in cultivation method types I and III, the content of gentiopicroside in stem and flower were significantly influenced by growing age (P<0.05), respectively. Plants that had been grown for 2 years were higher than that grown for 3 years.

At the same growing age, contents of gentiopicroside in root, stem, leaf and flower were less influenced by cultivation method.

Effect of various cultivations of swertiamarin content

The variation of swertiamarin contents of *G. rigescens* is listed in Table 4. The contents of swertiamarin in stem and leaf were significantly influenced by growing age

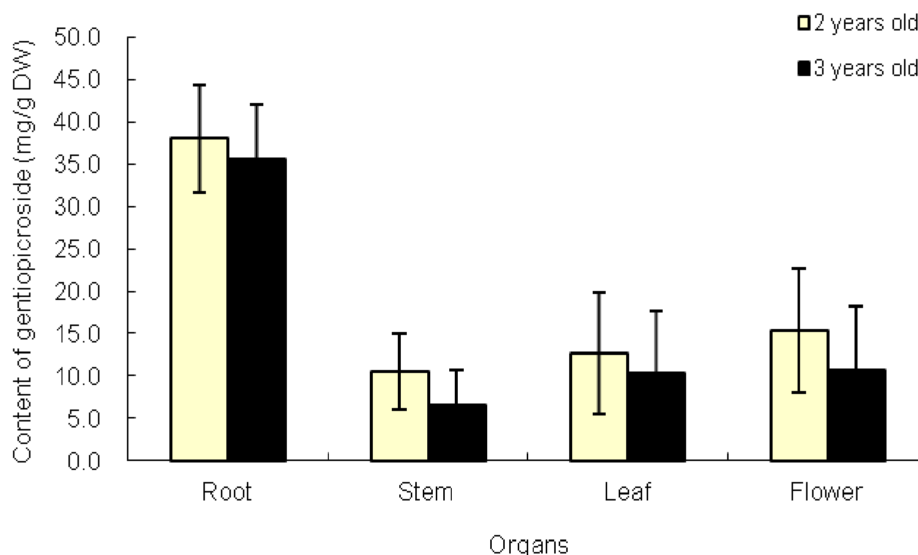

Figure 2. Content of gentiopicroside in different organs of *G. rigescens*.

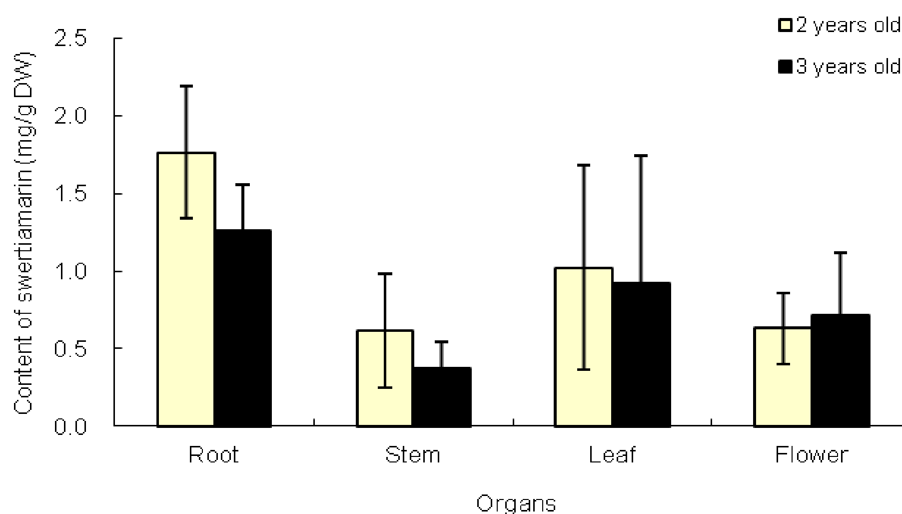

Figure 3. Content of swertiamarin in different organs of *G. rigescens*.

(P<0.05). In the cultivation types I and III, swertiamarin content in stem which was grown for 2 years was significantly higher than grown for 3 years (P<0.05). In the cultivation types II and III, swertiamarin content in leaf which was grown for 2 years were observably higher than that grown for 3 years (P<0.05). At the same growing age, content of swertiamarin in the plant was less influenced by cultivation method.

Effect of various cultivations of sweroside content

The variation of sweroside contents of *G. rigescens* is presented in Table 5. Except cultivation type III, growing age had no significant influence on sweroside content (P>0.05). In the cultivation type III, content of sweroside in leaf that had been grown for 2 years was higher than that grown for 3 years (P<0.05).

At the same growing age, content of sweroside in leaf of *G. rigescens* was significantly influenced by cultivation method (P <0.05). In type III cultivation method, content of swertiamarin in leaf which was grown for 2 years was the highest.

DISCUSSION

Secoiridoids as an important secondary metabolites were

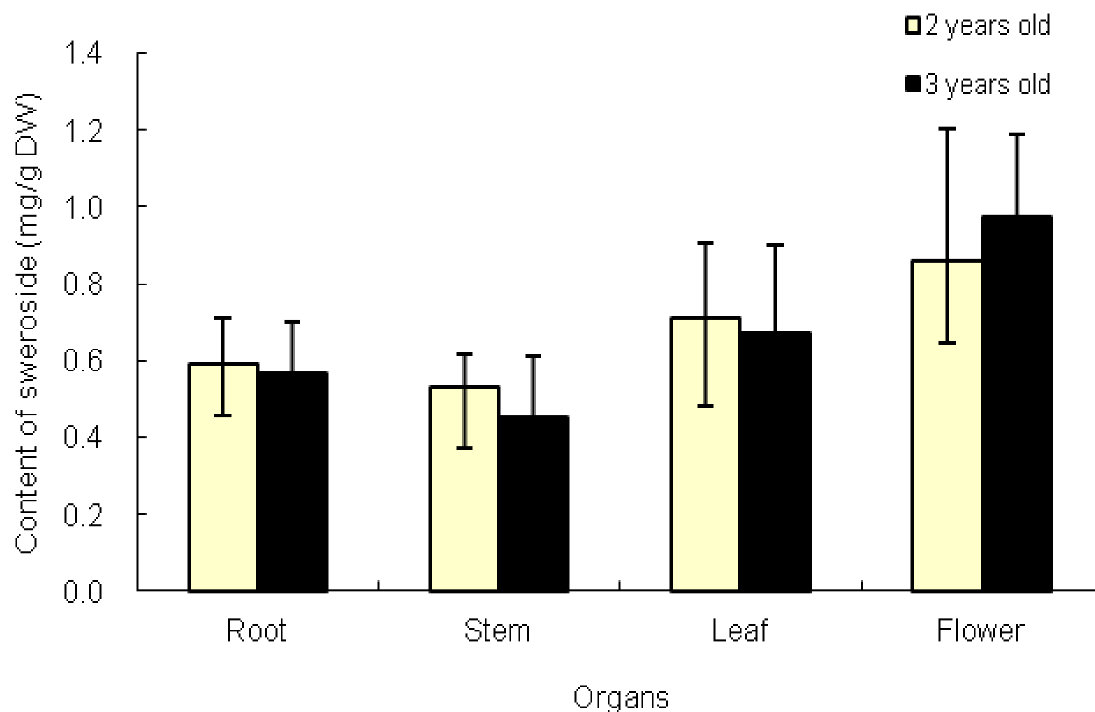

Figure 4. Content of sweroside in different organs of *G. rigescens*.

Table 3. Content of gentiopicroside in different organs of *G. rigescens* (mg/g).

Cultivation method	Root		Stem		Leaf		Flower	
	2 years	3 years	2 years	3 years	2 years	3 years	2 years	3 years
Type I	40.110 ±6.056aA	35.156±6.930aA	9.976±4.309aB	2.804±2.186aA	10.730±8.063aA	10.464±9.508aA	11.249±8.233aA	6.766±4.276aA
Type II	38.406±6.002aA	32.550±9.552aA	8.205±4.032aA	5.098±1.233aA	11.392±5.199aA	6.191±2.476aA	14.603±3.377aA	10.003±2.599aA
Type III	35.498± 7.353aA	38.730± 4.784aA	13.468±4.293aA	9.415±5.456aA	16.316±9.011aA	7.862±7.711aA	21.098±6.564aB	8.974±6.317aA

Different capital letters in each row indicating significant difference according to T- test (0.05 level) shows the effect of growing age; different small letters in each column indicating significant difference according to one-way ANOVA test (0.05 level) show the effect of cultivation method.

were isolated and widely reported from medicinal plants (Noungoué et al., 2001; Aljančić et al., 2008; Yao et al., 2008; Si et al., 2009; Silva et al., 2010). However, the research about relationship between secoiridoids content in genus *Gentiana* medicinal plant and cultivation condition was limited.

The present study had a similar result with *Gentiana triflora*, another medicinal plant cultivated

Table 4. Content of swertiamarin in different organs of *G. rigescens* (mg/g).

Cultivation method	Root		Stem		Leaf		Flower	
	2 years	3 years	2 years	3 years	2 years	3 years	2 years	3 years
Type I	1.913±0.629^{aA}	1.430±0.363^{aA}	0.405±0.143^{aB}	0.225±0.075^{aA}	0.700±0.327^{aA}	2.343±0.886^{bB}	0.598±0.160^{aA}	0.735±0.477^{aA}
Type II	1.656±0.175^{aA}	1.172±0.415^{aA}	0.564±0.382^{aA}	0.535±0.221^{bA}	0.777±0.224^{aB}	0.133±0.053^{aA}	0.573±0.178^{aA}	0.940±0.550^{aA}
Type III	1.721±0.393^{aA}	1.299±0.233^{aA}	0.927±0.405^{aB}	0.391±0.172^{abA}	1.643±0.900^{aB}	0.306±0.162^{abA}	0.724±0.354^{aA}	0.427±0.204^{aA}

Different capital letters in each row which indicate significant difference according to T- test (0.05 level) shows the effect of growing age; different small letters in each column which indicate significant difference according to one-way ANOVA test (0.05 level) show the effect of cultivation method.

Table 5. Content of sweroside in different organs of *G. rigescens* (mg/g).

Cultivation method	Root		Stem		Leaf		Flower	
	2 years	3 years	2 years	3 years	2 years	3 years	2 years	3 years
Type I	0.612±0.072^{aA}	0.643±0.196^{aA}	0.544±0.086^{aA}	0.465±0.011^{aA}	0.747±0.244^{abA}	0.679±0.254^{aA}	1.132±0.369^{aA}	0.982±0.056^{aA}
Type II	0.501±0.029^{aA}	0.609±0.153^{aA}	0.462±0.008^{aA}	0.549±0.057^{aA}	0.571±0.046^{aA}	0.519±0.208^{aA}	0.670±0.165^{aA}	0.915±0.294^{aA}
Type III	0.657±0.165^{aA}	0.477±0.027^{aA}	0.551±0.100^{aA}	0.335±0.260^{aA}	0.896±0.102^{bB}	0.552±0.033^{aA}	0.715±0.254^{aA}	0.919±0.193^{aA}

Different capital letters in each row indicating significant difference according to T- test (0.05 level) shows the effect of growing age; different small letters in each column indicating significant difference according to one-way ANOVA test (0.05 level) show the effect of cultivation method.

cultivated in north of tween secoiridoids content in genus *Gentiana* medicinal plant and cultivation condition was limited.

The present study had a similar result with *Gentiana triflora*, another medicinal plant cultivated in north of China.

They have same distribution of gentiopicroside and swertiamarin in plant organ, and root was the main store organ of gentiopicroside (Jiang, 2005a, b). Root of *G. triflora* and *G. rigescens* have a likely pharmacology. The two species have different distribution area but their likely pharmacology might relate to similar distribution of gentiopicroside.

The present research showed that content of secoiridoids in root of *G. rigescens* from Lincang was slightly influenced by cultivation condition, and plants grown for 2 years could reach the requirement of the active contents in China (Committee for the Pharmacopoeia of P.R. China, 2010). It suggested cultivation condition might affect yield of the root of *G. rigescens*. Although root was the traditional medicinal part of *G. rigescens*, the result indicated that aerial part of *G. rigescens* could be used instead of thrown away. The variation of chemical composition in stem and leaf could be affected by environmental factor. This problem will need to be researched further.

Conclusion

The contents of gentiopicroside, swertiamarin and sweroside in *G. rigescens* were varied with the change of organ and cultivation condition. The quality of root was stable. Cultivation had no significant effect on the quality of *G. rigescens* root, but had strong impact on the aerial part of the plant

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Full Length Research Paper

In vitro synergy and time-kill assessment of interaction between kanamycin and metronidazole against resistant bacteria

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This study assessed the influence of combining kanamycin and metronidazole against Gram positive and Gram negative bacteria by agar diffusion, checkerboard and time-kill assays. The test isolates were highly resistant with minimum inhibitory concentrations (MICs) ranging between 15.63 and >250 µg/ml for kanamycin and between 15.63 and 125 µg/ml for metronidazole. The antibacterial combinations resulted in drastic decreases in the MICs with an increased antibacterial activity that indicated synergistic interaction against all the bacteria except *Acinetobacter calcaoceuticus* UP, *Enterobacter cloacae* ATCC 13047 and *Shigella flexneri* KZN. The fractional inhibitory concentration indices (FICIs) showed synergy ranging from 0.31 to 0.50, additive interaction with FICI ranging from 0.53 to 1.25 and no antagonistic interaction. *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10702, *E. cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 10031, *A. calcaoceuticus* UP and *Micrococcus luteus* were totally eliminated by the antibacterial combinations within 24 h of incubation. The lack of antagonism between these antibacterial agents in checkerboard and time-kill assays suggests that kanamycin may prove to be effective in monotherapy and combination therapy. The study indicates the potential beneficial value of combining kanamycin and metronidazole in the treatment of microbial infections in clinical settings.

Key words: Drug-drug interactions, synergy, time-kill, FICI, microbial infections.

INTRODUCTION

Infectious diseases are a significant cause of morbidity and mortality accounting for approximately 50% of all deaths in tropical countries (Mahady, 2005; Khosravi and Behzadi, 2006) and a leading cause of death worldwide (Ahmad and Aqil, 2009). Due to indiscriminate use of antibacterial agents in infectious diseases (Davies, 1994; Service, 1995), multidrug resistance in bacteria has become a great challenge to human health (Peters et al., 2008). With the increasing prevalence of multi-drug resistant bacteria and appearances of strains with reduced susceptibility to antibiotics (Boucher et al., 2009) as well as the inexorable invasion of hospitals and communities, there are increases in health care costs (Gums, 2002), many untreatable bacterial infections and the need to search for new infection-fighting strategies and novel antibacterial agents (Zy et al., 2005; Rojas et al., 2006; Ymele-Leki et al., 2012).

Although previous studies have indicated interactions between other aminoglycosides or nitroimidazole (metronidazole) and other antibacterial agents, combining kanamycin and metronidazole against bacteria of clinical importance has not been reported. For the aminoglycosides,

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Burgess and Hastings (2000), Song et al. (2003) and CLSI (2006) reported the effectiveness of combining β-lactam with aminoglycoside in the treatment of *Pseudomonas aeruginosa*. Kelesidis et al. (2008) and Petrosillo et al. (2008) showed that polymyxins and tigecycline were combined for treating infections caused by carbapenem-resistant Enterobacteriaceae and Cottagnoud et al. (2003) reported the synergy of vancomycin and gentamicin against penicillin-resistant pneumococci. While synergistic clinical efficacies of amoxicillin and metronidazole have been reported (Winkel et al., 2001; Yek et al., 2010), Pulkkinen et al. (1993) and Sanchez et al. (2004) showed that metronidazole-nystatin combination produced better prevention against bacterial vaginosis in women using intra uterine drug (IUD) as a contraceptive method than the respective drug. Azithromycin combined with metronidazole was more effective in treating pelvic inflammatory diseases (Bevan et al., 2003), symptomatic bacterial vaginosis (Schwebke and Desmon, 2007) and pediatric Crohn's disease (Levine and Turner, 2011). The use of combinations of antimicrobials that together achieve synergistic activities against targeted micro-organisms is one potential strategy for overcoming bacterial resistance (Allen et al., 2002). Theoretically, it is aimed at broadening antimicrobial empirical coverage, improving efficacy against isolates with a minimum inhibitory concentration (MIC) at or approaching the breakpoint for susceptibility as well as preventing the further emergence of resistant organisms (Rybak and McGrath, 1996; Walsh and Howe, 2002). Drug combinations are characterized by an increased activity and tolerability compared to that of monotherapy and those used to increase the killing of single-drug resistant strains or mutants. While preventing the emergence of reduced susceptibility, it achieves bactericidal synergy and provides activity against stationary-phase organisms and organisms growing in biofilm. The use of drug combinations is an excellent strategy to avoid or delay drug resistance since different drug targets are attacked simultaneously. This study was, therefore, aimed at assessing the effect of combining kanamycin and metronidazole, having different mechanisms of action, against bacteria of clinical relevance.

MATERIALS AND METHODS

Bacterial strain

The bacteria used in this study included *Acinetobacter calcaoceuticus* UP, *Bacillus cereus* ATCC 10702, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 10031, *Shigella flexneri* KZN, *Micrococcus luteus, E. faecalis* KZN and *Staphylococcus aureus* OK_{2b}. These organisms were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The antibacterial assays were carried out using Mueller Hinton II Agar (Biolab) and broth.

Antibiotics used in this study

Antibiotic powders of Kanamycin (Duchefa) and Metronidazole (Duchefa) were used. Stock antibiotic solutions were prepared and dilutions made according to the CLSI (Clinical Laboratory Standardization Institute) method or manufacturer's recommendations (NCCLS, 1997; Richard et al., 2007).

Antibiotic susceptibility testing - (Agar diffusion method)

Each of the isolates was standardized using colony suspension method (EUCAST, 2012). Each strain's suspension was matched with 0.5 McFarland standards to give a resultant concentration of 1 × 10^6 cfu/ml. The antibiotic susceptibility testing was determined using the modified Kirby-Bauer diffusion technique (Cheesbrough, 2002) by swabbing the Mueller-Hinton agar (MHA) (Oxoids U.K) plates with the resultant saline suspension of each strain. Wells were then bored into the agar medium with heat sterilized 6 mm cork borer. The wells were filled with 100 µl of different concentrations (62.5, 125 and 250 µg/ml) of each of the antibiotics taking care not to allow spillage of the solutions onto the surface of the agar. To determine the combinatorial effect of the antibiotics, different solutions containing combined concentrations (62.5, 125 and 250 µg/ml) of kanamycin and metronidazole were prepared and used. The plates were allowed to stand for at least 30 min before being incubated at 37°C for 24 h (BSAC, 2002). The determinations were done in duplicate. After 24 h of incubation, the plates were examined if there is any zone of incubation (Bauer et al., 1966). The diameter of the zone of inhibition produced by the respective antibiotic alone and their combinations were measured and interpreted using the CLSI zone diameter interpretative standards (CLSI, 2008).

Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) for the two antibiotics under study were determined in duplicate by the macrobroth dilution method in Mueller Hinton broth according to CLSI (Clinical Laboratory Standardization Institute) (Richard et al., 2007). To determine the MICs of each antibiotic, different concentrations of each of the antibiotics (0.0019 - 500) µg/ml were prepared by serial dilution in Mueller Hinton broth. To determine their combinatorial effects, combinations of different concentrations used in the determination of the MICs of each of the antibiotics were used. The tubes were inoculated with 100 µl of each of the bacterial strains. Blank Mueller Hinton broth was used as negative control. The bacterial containing tubes were incubated at 37°C for 24 h. Each combination assay was performed two times. The MIC was defined as the lowest dilution that showed no growth in the Mueller Hinton broth.

Checkerboard assay

The interactions between the two antibiotics were determined using the checkerboard as previously described (Petersen et al., 2006). The range of drug concentration used in the checkerboard assay was such that the dilution range encompassed the MIC for each drug used in the analysis. The fractional inhibitory concentration (FIC) was derived from the lowest concentrations of the two antibiotics in combination permitting no visible growth of the test organisms in the Mueller Hinton broth after an incubation for 24 h at 37°C (Mandal et al., 2004). FIC indices were calculated using the formula FIC index = (MIC of kanamycin in combination/MIC of kanamycin alone) + (MIC of metronidazole in combination/MIC of metronidazole alone). While Eliopoulos and Eliopoulos (1988),

Isenberg (1992) and Petersen et al. (2006) defined synergy by the checkerboard method as $\sum FIC \leq 0.5$, additivity as $0.5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$ and antagonism as $\sum FIC > 4$, Giertsen et al. (1988), Grytten et al. (1988), and Kamatou et al. (2006) defined synergy to occur when $\sum FIC < 1.0$, additivity occur when $\sum FIC = 1.0$ and antagonism when $\sum FIC > 1.0$. In this study, synergy was defined as $\sum FIC \leq 0.5$, additivity as $0.5 < \sum FIC \leq 1$, indifference as $1 < \sum FIC \leq 4$. Concentrations within the FIC panel were such that the MIC of each antibiotic was in the middle of the range of concentrations tested but lower than the MICs of the respective antibiotics.

Determination of rate of kill

Assays for the rate of killing bacteria by the combined antibiotics were carried out using a modified plating technique of Eliopoulos and Eliopoulos (1988) and Eliopoulos and Moellering (1996). The combined antibiotics were incorporated into 10 ml Mueller Hinton broth in McCartney bottles at ½ MIC and MIC. Two controls, one Mueller Hinton broth without combined antibiotics inoculated with test organisms and Mueller Hinton broth incorporated with the combined antibiotics at the test concentrations without the test organisms, were included. Inoculums density, approximately 10^{10} cfu/ml further verified by total viable count, was used to inoculate 10 ml volumes of both test and control bottles. The bottles were incubated at 37°C on an orbital shaker at 120 rpm. A 100 µl aliquot was removed from the culture medium at 0, 24 and 48 h for the determination of cfu/ml by the plate count technique (Cruishank et al., 1975) by plating out 25 µl of each of the dilutions. The problem of antibiotics carryover was addressed by dilution as described previously by Pankuch et al. (1994). After incubating at 37°C for 24 h, emergent bacterial colonies were counted, cfu/ml calculated and compared with the count of the culture control without antibiotic.

RESULTS

In this study, *A. calcaoceuticus* UP and *E. faecalis* KZN were highly resistant to kanamycin while other isolates exhibited concentration dependent susceptibility to the varied concentrations of this antibiotic. To metronidazole, *E. faecalis* KZN was susceptible to the different concentrations, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 10031 and *A. calcaoceuticus* UP were slightly inhibited by the highest concentration. Other isolates were not affected by the different concentrations used. On combining different concentrations of the two antibiotics, concentration dependent significant synergistic interactions were observed. The resultant zones of inhibition from the combined antibiotics were wider than those obtained from the antibacterial activities of each antibiotic used (Table 1). Though the bacteria showed varied resistance to both antibiotics, resistant colonies were not isolated within the zones of inhibition and fuzzy zones were not found around the edges of the zones of inhibition.

The macrobroth assay indicated that the test isolates were highly resistant to the two antibiotics by exhibiting minimum inhibitory concentrations (MICs) ranging between 15.625 and >250 µg/ml for kanamycin and 15.625 to 125 µg/ml for metronidazole. On combining the two antibiotics against these bacteria, the MICs of both antibiotics were drastically reduced in the range between ½ MIC and $^{1}/_{8}$ MIC with a simultaneous increase in the antibacterial activity of the combined antibiotics (Table 2). The results of both assays were complementary. The significant reduction in the MICs and the observed increase in the zones of inhibition from combined antibiotics showed that the resultant effect of combining these two antibiotics was synergy. In the checkerboard assay, combining the two antibiotics showed synergistic interaction against most of the bacteria except *Acinetobater calcaoceuticus* UP, *E. claoacae* (ATCC 13047) and *S. flexneri* KZN. While the fractional inhibitory concentration indices (FICIs) showing synergy ranged from 0.3125 – 0.5, an additive/indifference interaction was indicated with FICI ranging between 0.5313 and 1.25 and no antagonism was recorded from the antibacterial combinations. The combined antibiotics indicated ability to improve the bactericidal effects of each other on both Gram-negative and Gram-positive bacteria.

In the time-kill assay, the results presented in terms of the changes in the $\log_{10}$ cfu/ml of viable colonies showed that the antibacterial combinations exhibited a significant bactericidal activity. The bactericidal activity was defined as being equal to 3 $\log_{10}$ cfu/ml or greater reduction in the viable colony count relative to the initial inoculum (Scheetz et al., 2007). *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *B. cereus* ATCC 10702, *E. cloacae* ATCC 13047 and *M. luteus* were completely annihilated by the combination of kanamycin and metronidazole at ½ MICs. In addition to these bacteria, *K. pneumoniae* (ATCC 10031) and *A. calcaoceuticus* UP were totally killed by the combined activity of both antibiotics at the MICs within 24 h of incubation. *S. flexneri* KZN, *E. faecalis* KZN and *Staphylococcus aureus* OK_{2b} were not totally inhibited at the combined MICs, despite the degree of the observed synergism because each of these isolates exhibited a very high level of resistance to either or both antibiotics. Average log reduction in viable cell count in time-kill assay for *K. pneumoniae* ATCC 10031, *A. calcaoceuticus* UP, *S. flexneri* KZN, *E. faecalis* KZN and *Staphylococcus aureus* OK_{2b}, not totally eliminated, however, ranged between 3.4472 Log_{10} to 5.7782 Log_{10} cfu/ml after 24 h of interacting the bacteria with the combined antibiotics at the ½ MIC and MIC values (Table 3). A post-antibiotic treatment bioassay done after 48 h showed that all isolates not totally inhibited within 24 h incubation period had an increase in cfu/ml.

DISCUSSION

Due to the frequent development of resistance during monotherapy treatment of infected patients, multiple combinations of antibacterial agents are being proposed (Campbell et al., 1996; El Solh and Alhajhusain, 2009). This is to effectively treat mixed and severe infections,

Table 1. Zones of inhibition produced by each antibiotic and their combinations at different concentrations.

Zone of inhibition	Kanamycin alone (± 1.0 mm)			Metronidazole (± 1.0 mm)			Kan-Met combinations (± 1.0 mm)		
	250	125	62.5	250	125	62.5	250/250	125/125	62.5/62.5
	(µg/ml)			(µg/ml)			(µg/ml)		
Escherichia coli ATCC 25922	25	20	18	0	0	0	29	26	20
Enterococcus faecalis ATCC 29212	26	24	21	16	0	0	27	24	21
Bacillus cereus ATCC 10702	27	24	22	0	0	0	29	25	23
Enterobacter cloacae ATCC 13047	22	20	19	0	0	0	25	22	20
Klebsiella pneumoniae ATCC 10031	28	25	21	13	0	0	29	27	22
Acinetobacter calcaoceuticus UP	0	0	0	14	0	0	26	24	20
Shigella flexneri KZN	28	25	23	0	0	0	28	26	22
Micrococcus luteus	24	21	20	0	0	0	28	25	21
Enterococcus faecalis KZN	0	0	0	20	19	14	31	28	23
Staphylococcus aureus OK_{2b}	28	24	22	0	0	0	26	24	20

Table 2. Fractional inhibitory concentration values for the antibiotics alone and their combinations against resistant bacterial isolates.

Tested bacteria	Minimum inhibitory concentration (µg/ml)			Fractional inhibitory concentration index			
	Kanamycin	Metronidazole	KAN-MET	FICI Kan	FICI Met	FICI	Remarks
Escherichia coli ATCC 25922	125	31.25	15.63/7.81	0.13	0.25	0.38	Synergistic
Enterococcus faecalis ATCC 29212	125	31.25	15.63/7.81	0.13	0.25	0.38	Synergistic
Bacillus cereus ATCC 10702	125	31.25	7.81/7.81	0.06	0.25	0.31	Synergistic
Enterobacter cloacae ATCC 13047	62.5	31.25	15.63/15.63	0.25	0.5	0.75	Additive
Klebsiella pneumoniae ATCC 10031	31.25	31.25	7.81/7.81	0.25	0.25	0.5	Synergistic
Acinetobacter calcaoceuticus UP	> 250	15.63	7.81/7.81	0.03	0.5	0.53	Additive
Shigella flexneri KZN	15.63	62.25	15.63/15.63	1.0	0.25	1.25	Indifference
Micrococcus luteus	250	31.25	15.63/7.81	0.06	0.25	0.31	Synergistic
Enterococcus faecalis KZN	> 250	62.5	15.63/15.63	0.06	0.25	0.31	Synergistic
Staphylococcus aureus OK_{2b}	62.5	125	15.63/15.63	0.25	0.125	0.38	Synergistic

enhance antibacterial activity, reduce the time needed for long-term antimicrobial therapy and prevent the emergence of resistant micro-organisms (Hugo, 1993; Levinson and Jawetz, 2002). Combining existing antimicrobial agents can improve delivery of safe and cost effective patient care in an era where research into discovery of new agents is limited and expensive. In this study, examination of synergy by the checkerboard method demonstrated synergy between kanamycin and metronidazole for the majority of the strains while antagonism was not observed. This is in agreement with previous reports on interaction between aminoglycosides and other antibacterial agents (Tessier and Quentin, 1997; Hayami et al., 1999). Their combination in chemotherapy could decrease resistance development, broaden antibacterial spectrum and

Table 3. In vitro time-kill activity of Kanamycin – Metronidazole combinations at ½ X MIC and MIC against test bacteria.

Tested bacteria	Reduction in bacterial counts (Log_{10}CFU/ml) for the combined antibiotics					
	½ × MIC			MIC		
	0 h	24 h	48 h	0 h	24 h	48 h
Escherichia coli ATCC 25922	7.18	0	0	6.90	0	0
Enterococcus faecalis ATCC 29212	8.90	0	0	8.91	0	0
Bacillus cereus ATCC 10702	7.62	0	0	7.26	0	0
Enterobacter cloacae ATCC 13047	12.15	0	0	12.38	0	0
Klebsiella pneumoniae ATCC 10031	11.51	5.78	5.97	11.61	0	0
Acinetobacter calcaoceuticus UP	12.70	3.45	3.94	12.81	0	0
Shigella flexneri KZN	12.43	3.92	4.32	12.51	3.86	3.96
Micrococcus luteus	11.93	0	0	11.99	0	0
Enterococcus faecalis KZN	10.42	4.11	4.54	11.15	3.90	4.28
Staphylococcus aureus OK_{2b}	9.38	5.13	5.27	9.66	3.94	4.08

encourage synergistic antibacterial activity (den Hollander and Mouton, 2007). As determined by Eliopoulos and Moellering (1996), antibiotic combinations that reduced the original inoculums by ≥ 2 log_{10} cfu/ml were considered synergy while antagonsism is a < 2 log_{10} change in cfu/ml when compared with the activity of the individual antibiotic after 24 h incubation period. The time-kill assay confirmed the synergy between kanamycin and metronidazole as indicated by the checherboard assay. These synergy that resulted in enhance antibacterial effects from antibiotics having different mechanisms of action could have resulted from the formation of a complex compound with enhanced antibacterial activity. Since kanamycin prevents bacteria from synthesizing proteins by binding to 16S rRNA of 30S subunit and metronidazole is reduced to cytotoxic polar compounds able to cause DNA strand breakage, DNA helix and nucleic acid destabilization in bacteria (Tocher and Edwards, 1992; 1994), the activity of the antibacterial combination could be complementary and resulted in the rapid death of bacterial colonies. This could be a means of achieving effective therapy at a reduced cost coupled with a drastic reduction or lost of vestibular and auditory toxicity often associated with the aminoglycosides. While the lack of antagonism between the antibiotics suggested that kanamycin or metronidazole may be effective in monotheryapy and combination therapy, the resultant synergy will reduce the dose of each drug in the combination and prevents the development of bacterial resistance (Barriere, 1992; Wu et al., 1999).

Although bactericidal drugs prevent the emergence of resistant mutants by killing the microorganism (Stratton, 2003) while synergy and bactericidal therapy could be achieved as long as the organism does not exhibit high-level resistance to aminoglycoside (Arias and Murray, 2008), it is evident that highly resistant bacteria with MIC ranging between 15.625 and >250 µg/ml for kanamycin were killed by its combination with metronidazole to which the MICs were between 15.625 and 125 µg/ml for the different isolates. However, while Mouton et al. (1997) and Jumbe et al. (2003) have conceptualized a microbial population as consisting of two distinct subpopulations with different susceptibility, the regrowth of *S. flexneri* KZN, *E. faecalis* KZN and *Staphylococcus aureus* OK_{2b} could be attributed to the preferential killing of the susceptible subpopulations allowing the selective increase of the resistant subpopulation of each of these resistant strains after 48 h incubation. Further treatment or subsequent doses of the antibacterial combinations would be sufficient to eliminate the resistant subpopulation.

Conclusion

In clinical settings, this study emphasizes the potential beneficial value of combining kanamycin and metronidazole for treating seriously ill patients with infections caused by the pathogens tested, especially in the absence of other therapeutic options. Future studies in *in vivo* infection models would provide a better understanding of the therapeutic potential and safety of kanamycin-metronidazole combinations.

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Full Length Research Paper

Analysis on 102 cases of adverse reactions caused by amoxicillin

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To analyze the reports of adverse reactions caused by amoxicillin and explore its occurrence rules and characteristics. To collect the cases of adverse reactions caused by amoxicillin in the past five years in the hospital and make the statistical analysis. In the reports of the 102 cases of adverse reactions, the clinical manifestations were mainly skin and its accessory damage and systemic damage. Allergic reaction is the most common clinical manifestation of the adverse reactions caused by amoxicillin, and physicians should pay attention to patient's history of drug allergy and make close observation at the first drug use, which has important significance in prevention of the adverse reactions caused by amoxicillin.

Key words: Amoxicillin, adverse reactions, preventive measures.

INTRODUCTION

Amoxicillin is a β-lactam antibiotic, and oral dosage which form has good absorption, easy use and broad spectrum used for respiratory tract infection, urinary tract infection, ear, nose, throat, skin and soft tissue infections, and typhoid fever. While recognizing the efficacy of this drug, people neglect its adverse reactions. This paper collects and analyses the statistics on the cases of adverse reactions caused by this antibiotic in the past five years in our hospital.

DATA AND METHODS

Epidemiological analysis of the survey showed that the incidence of adverse reactions was 21.93% (the ADRs center of Beijing in China), which has attracted our attention (Qi-ping Liu et al., 2001). 102 cases of adverse reactions caused by amoxicillin were collected from outpatients and some inpatients with age of 50 days – 80 years old in our hospital in 2005-2010. Classification was made according to the adverse reaction types specified by WHO Adverse Drug Reaction Monitoring Center. Statistics and analysis were also made on age, gender, primary diseases, drug usage, clinical manifestations of adverse reactions and prognosis, and the relevant characteristics of adverse reaction occurrence was discussed.

RESULTS

Age and gender

In 43 males and 59 females with an age range of 50 days-80 years old, 18 cases have past drug allergy history, 20 cases have no drug allergy history, and the other 64 cases have unknown drug allergy history, as shown in Table 1. Primary diseases are shown in Table 2.

Drug usage, adverse reactions and clinical manifestations

Amoxicillin has better gastrointestinal absorption, so most

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Table 1. Distribution of ages and gender.

Age	Male	Female	Total
<20	14	12	26
20-39	11	19	30
40-59	10	18	28
60-79	5	6	11
>80	3	4	7
Total	43	59	102

Table 2. Distribution of primary diseases.

Primary disease	n	Proportion (%)
Endocarditis	12	11.76
Gastritis	23	22.54
Peptic ulcer	9	8.82
Acute tonsillitis	28	27.45
Breast lobule hyperplasia	16	15.69
Influenza	8	7.84
Others	6	5.88
Total	102	100

patients orally take this drug. Some patients also choose injection, as the efficacy comes faster. In addition, amoxicillin is often combined with other drugs, such as Chinese patent drugs, antibacterial drugs, antipyretic and analgesic drugs, digestive system drugs, respiratory system drugs, vitamin drugs, etc. There are 25 cases of patients with rational use of drugs (Table 3). The listed items have no obvious incompatibility with the amoxicillin (Xiao-yun et al., 2007). Different types of adverse reactions and clinical manifestations are shown in Table 4.

Prognosis of adverse reactions caused by amoxicillin

The adverse reactions caused by amoxicillin were relieved or the intensity decreased after stopping the drug administration, by symptomatic antiallergic and other kinds of treatments. Only few cases of allergic shock died after rescue.

DISCUSSION

Relationship between ages and gender and adverse reactions

It can be seen from the statistical result that the ages of patients with adverse reactions caused by amoxicillin are widely distributed, but mainly at the ages of 20-60 years old. For gender, female is slightly higher than male, probably because the incidence of gynecological inflammation in female is higher.

Relationship between primary diseases and adverse reactions

For the types of primary diseases, the patients generally suffer from respiratory tract infection, gastrointestinal tract infection, urinary tract infection, etc, which are related with pyogenic streptococcus, pneumococcus, hemophilus influenzae, Escherichia coli, pneumobacillus and other Gram-positive and Gram-negative bacteria, indicating that amoxicillin has high antibacterial activity against Gram-positive and Gram-negative bacteria (Ju-fang et al., 1999) .

Relationship between drug combination and adverse reactions

Rational drug combination therapy can increase efficacy. For example, amoxicillin combined with lincomycin and erythromycin has better efficacy in treatment of endocarditis. In addition, amoxicillin combined with omeprazole can effectively remove *Helicobacter pylori* (Kucers et al., 2010). But, mis-combination will cause serious adverse reactions. A related literature reports that administration of gentamicin sulfate, houttuynia cordata injection and amoxicillin for a patient with acute enteritis caused papules and limb convulsion, and excluding the allergy to other drugs, the patient was accurately diagnosed as allergy to amoxicillin. After a patient with duodenal ulcer orally took amoxicillin and Losec, low platelet level occurred (Qi-ping, 2003). After treatment of peptic ulcer with the combination of clarithromycin, omeprazole and amoxicillin, nausea, vomiting, diarrhea, constipation and other adverse reactions occurred (Yun-jian, 2010). After combination therapy of amoxicillin and potassium clavulanate, liver toxic reaction, hemorrhagic enteritis, abscess and child tooth pigmentation also occurred (Zhong-shan et al., 2004; Garcia et al. 2003).

Therefore, drug combination should be used with caution, medical care staff should make surveys before drug use and carefully ask allergy history and family history. Patients should also cooperate actively and tell the disease conditions and drug taking.

Clinical manifestations of adverse reactions

It can be seen from Table 4 that the clinical manifestations of adverse reactions caused by amoxicillin is firstly observed on the skin and secondly in the digestive

Table 3. Combination therapy in 25 cases of amoxicillin adverse reactions.

Class of drugs	Combined drug
Antimicrobial agents	Metronidazole tablets, Gentamicin injection, Amikacin injection , Cephradine injection
Antipyretic and analgesic	Paracetamol tablets, Aspirin tablets, Quike capsule
Chinese patent medicines	Small children-speed efficiency flu tablets, LingCao coral buccal tablet, Compound Liquorice Tablets, Isatidis granules, Changyanning Tablet, anti-cold and heat-clearing electuary, detoxicating tablet of cow-bezoar, jizhi syrup, Voiceless pill, tablet for treating common cold, cholagogic tablet, Wenweishu granules, Breast Mass Resolving Tablet, Shuanghuanglian oral liquor, Houttuynia cordata injection, Powerful VC yinqiao tablets
Digestive System Drugs	Losec capsules, Omeprazole, Lansoprazole tablets, Famotidine tablets
Respiratory medicine	Aminophylline tablets
Vitamins	Vitamin B 6 tablets

Patients with more than two kinds of medication, so the combined number of drugs is greater than 25.

Table 4. Types and clinical manifestations of adverse reactions.

Adverse reaction type	Clinical manifestations	n	Proportion (%)
Skin	Drug rash, drug fever, herpes, exfoliative dermatitis	25	24.5
Digestive system	Nausea, vomiting, abdominal pain, watery stool	15	14.7
Nervous system	Exciting, dysphoria, tinnitus, deafness	5	4.9
Blood system	Thrombocytopenia, allergic purpura	4	3.92
Urinary system	Frequent urination, hematuria, proteinuria, acute interstitial nephritis	7	6.86
Respiratory system	Difficult breathing, asthma, laryngeal edema	10	9.8
Systemic damage	Death caused by shock, allergic shock, syncope	17	16.67
Others	Lymph node enlargement, hemianopia, toe vein thrombosis	19	18.63
Total		102	100

organs. Additionally, some literatures report some rare adverse reactions, such as severe tinnitus, death caused by allergy, hemianopia, allergic shock complicated with hypoglycemic coma, hematuria (Jin-lan, 2010), toe vein thrombosis (Jun and Yu-ming, 2007), etc. Therefore, physicians or patients should be cautious in drug use, physicians should use drugs after completely learning about patient's conditions, and patients should carefully read the instructions before drug use and learn about drug application scope and adverse reactions after drug use.

Reasons for adverse reaction increase and the preventive measures

The reasons for adverse reaction increase are mainly as follows. First, patients buy and abuse antibiotics. As people's cultural level rising, the ability to accept new things is strong, but people know little about drugs, especially antibiotics, even though some are "illiterate". Many people mistakenly believe that more expensive drug is better. Second, drug stores improperly sell antibiotics, antibiotics supervision in China is not strict, and they can be bought in drug stores easily, which provides chances for antibiotic abuse. Third, medical care staff should strengthen the training for rational use of antibiotics, to improve their identification of indications for antibiotic use and ability for correct use of antibiotics. Fourth, economic factors also contribute to this problem, and free medical care system reform is urgent (Guang-ping, 2010; Ju-fang and Hui-ying, 1995; Kunin et al., 1990). The use of antibiotics should follow certain principles, thus it can effectively prevent the occurrence

of adverse reactions. First, patients diagnosed as bacterial infection can use antibiotics; second, to determine infectious agents and choose antibiotics according to the pathogenic species; third, drugs should be rationally chosen according to characteristics of drug effects, and those which can be used alone should not be used in combination, to reach the goal of good efficacy and little side effect; fourth, treatment options should be determined according to patient's condition, pathogen species and the characteristics of drug effects, and particularly the elderly and children should be cautious to use drugs. The tissues and organs of the elderly show degenerative changes in physiology, and immune function decreases, once they are infected, drugs will be metabolized slowly and more accumulated in body, thus causing adverse reactions, so the elderly should choose the antibiotics with low toxicity and good bactericidal effects. For children, their organs have not yet been developed well, especially liver and kidney function, and drugs are easily accumulate in the body, so they should choose the antibiotics with low liver toxicity and low kidney toxicity and that are easy to monitor by the plasma concentration (Niehols, 1995; Zhi-hui, 2010; Ji-ping et al., 2000). Only when stopping drug misuse and mastering drug use principles, we can ensure safe, rational and effective drug use and reduce the occurrence of adverse reactions.

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Full Length Research Paper

Combined treatment with resveratrol prevents the atorvastatin-induced myopathy in rat skeletal muscle

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Statins are widely used besides their myopathic side effects, ranging from mild myalgia to fatal rhabdomyolysis. Resveratrol is one of the most popular over the counter products used for similar purposes with statins. The aim of this study was to elucidate the myopathic effects of atorvastatin and coadministered resveratrol in male rat skeletal muscle via morphological analyses and immunohistochemistry studies. Control group received 1.5 ml of drinking water by oral gavage and 1 ml 15% ethanol (vehicle of resveratrol) i.p. for 14 days daily; atorvastatin group was treated with 40 mg/kg atorvastatin by oral gavage and 1 ml 15% ethanol i.p. for 14 days daily. Resveratrol + atorvastatin group was treated with 40 mg/kg atorvastatin by oral gavage and 20 mg/kg i.p resveratrol for 14 days daily. Atorvastatin treatment resulted with a moderate inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) immunoreactivity in nucleus and strong immunoreactivity in fibers. Control group and resveratrol + atorvastatin group showed weak iNOS and eNOS immunoreactivity in nucleus and moderate immunoreactivity muscular fibers. Treatment with atorvastatin resulted in a significantly shortened fibrils, and resveratrol co-treatment reversed this effect. Resveratrol and atorvastatin co-treatment could be an alternative treatment to prevent the myositis adverse effects of atorvastatin on skeletal muscle.

Key words: Atorvastatin, myositis, resveratrol.

INTRODUCTION

5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase reaction is the rate limiting step of cholesterol biosynthesis and thus the primary mechanism of action of HMG-CoA reductase inhibitors (statins) is to lower cholesterol levels (Evans and Rees, 2002). Statins are widely used in the prevention of cardiovascular events. Although they are generally well tolerated, different grades of myopathy, ranging from mild myalgia to fatal rhabdomyolysis has been reported (Abourjaily et al., 2003; Omar et al., 2002; Graham et al., 2004). The most serious risk associated with statins is myositis with rhabdomyolysis. The incidence of rhabdomyolysis has been estimated to be 0.44 to 0.54 cases per 10,000 person/years (Shek and Ferrill, 2001; Graham et al., 2004; Arora et al., 2006). The prevalence of milder muscle complaints like myalgia has been reported by statin

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users range as high as 20% (Foley et al., 2004; O'Meara et al., 2004; Buettner et al., 2008). Some authors have also reported the incidence of myalgia approximately in 5 to 7% of all patients on statins (Arora et al., 2006). Although the risk of rhabdomyolysis with currently marketed statins is very low, symptomatic muscle weakness and pain are much more frequent. Several possible mechanisms such as depletion of secondary metabolic intermediates, induction of apoptosis and alterations of chloride channel conductance within myositis have been proposed for statin-associated myopathy (Pierno et al., 2009). But the reason why myopathy develops in some patients as a result of statin treatment is still not well understood and this side effect prevents patients and their physicians from complying with statin therapy guidelines.

Hypercholesterolemic patients may sometimes direct to various dietary components and natural compounds to regulate serum lipid concentrations because of these doubts on the safety of statins. Resveratrol is one of the most popular over the counter (OTC) products used for similar purposes. Resveratrol (trans-3,5,4'-trihydroxystilben) is a polyphenol (phytoalexin) naturally found mostly in red wine and different therapeutic plants. By *in vitro* experiments, it has been shown that the cardiovascular protective effects of resveratrol might be through a variety of mechanisms such as inhibition of smooth muscle cells proliferation, platelet aggregation, and the oxidation of low-density lipoprotein (LDL) cholesterol. Resveratrol also reduces the synthesis of lipids and eicosanoids, which promote inflammation and atherosclerosis (Soner et al., 2010). Such multiple protective effects of resveratrol increase its demand as an OTC product even for statin users.

The present study was designed to elucidate the effects of combined treatment of resveratrol on atorvastatin-induced myopathy in male rat skeletal muscle via morphological analyses and immunohistochemistry studies.

MATERIAL AND METHODS

Animals and experimental protocol

Three groups of male Wistar-albino rats of 8 weeks, weighing 260 to 280 g were used in the experiments. Animals were housed identically in cages in an air conditioned room under a 12 h light dark cycle. Temperature and humidity were controlled within the limits 21 ± 2°C and 55 ± 15% relative humidity (RH). All animals became acclimatized for at least 7 days before the outset of the study. A standard diet and tap water were provided *ad libitum*. The experimental protocols were approved by the Animal Ethics Committee of Selcuk University, Meram Medical School. Control group received 1.5 ml of drinking water by oral gavage and 1 ml 15% ethanol (vehicle of resveratrol) i.p. for 14 days daily (n = 8); atorvastatin group was treated with 40 mg/kg atorvastatin (Lipitor®, prepared daily and dissolved in drinking water) by oral gavage and 1 ml 15% ethanol i.p. for 14 days daily (n = 6). Resveratrol + atorvastatin group was treated with 40 mg/kg atorvastatin by oral gavage and 20 mg/kg i.p resveratrol for 14 days daily (n = 8). Rats were weighted every 5 days to rearrange dosing schedule and observed every day or as necessary. When the body weight loss exceed 15% of day 1, rats were excluded from the study group. On the 15th day, the following muscle tissues were sampled: trapezius, gastrocnemius, semitendinosus and biceps femoris.

Tissue processing and immunohistochemistry

Paraformaldehyde fixation of tissues continued for 24 h, at 4°C and processed for embedding in paraffin wax using routine protocols. 5 µm-thick coronal sections were cut using a microtome (Leica MR 2145, Heerbrugg, Switzerland); they were then dewaxed and rehydrated through a graded ethanol series using routine protocols. Sections were then washed with distilled water and phosphate buffered saline (PBS) for 10 min, then treated with 2% trypsin (Sigma Chemical Co., St. Louis, Missouri, USA) in 50 mM Tris buffer (pH 7.5), at 37°C, for 15 min. Sections were delineated with a marker pen (Dakopen, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. To reduce non-specific background staining, slides were incubated at room temperature for 30 min in 0.3% bovine serum albumin/1 × Tris-buffered saline. Then, sections were incubated with primary antibodies directed against inducible nitric oxide synthase (iNOS) (1:100 dilution; Abcam, Cambridge, UK), endothelial nitric oxide synthase (eNOS) (1:200 dilution; Abcam, Cambridge, UK) for 18 h at 4°C in a humid chamber. Sections were then incubated with biotinylated secondary antibody and then with streptavidin conjugated to horseradish peroxidase (Histostain plus peroxidase kit, Zymed Laboratories Inc., South San Francisco, CA, USA) for 30 min in accordance with the manufacturer's instructions. Finally, sections were incubated with diaminobenzidine (DAB) for 5 min to reveal immunolabelling. All dilutions and thorough washes between stages were performed using PBS. Sections were counterstained with Mayer's hematoxylin (Sigma Chemical Co., St. Louis, Missouri, USA).

After washing with tap water, sections were dehydrated through a graded ethanol series, cleared in xylene and mounted with entellan. Negative control samples were processed as described except that primary antibodies were omitted and replaced with PBS alone. Positive controls were represented by sections of a neuroblastoma specimen known to be positive for the markers of interest. The intensity of iNOS and eNOS immunohistochemical stainings was graded semiquantitatively according to the nuclear and cytoplasmic immunoreaction in sections as follows: (-) no immunostaining, (+) weak staining, (++) moderate staining, (+++) strong staining. Light microscope, equipped with a camera (Olympus BX-51 and Olympus C-5050 digital camera, Olympus Co., Tokyo, Japan) was used. The slides were examined by two investigators.

Morphometrical analysis

Longitudinal sections were cut and stained with Gomori's trichrome. The first and second of the three consecutive serial sections were omitted and 12 sections from each subject were taken for quantification from the third. Preparations were screened systematically using a random start. Visualization of specimens at ×40 magnification was started from the top right corner of the preparation. Sections were examined under a light microscope

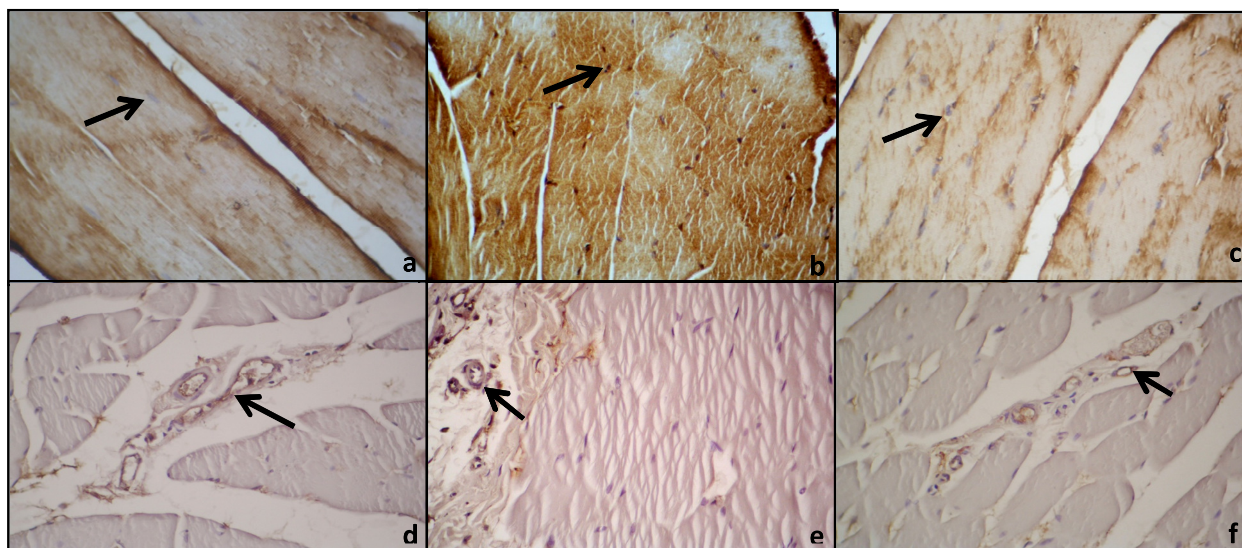

Figure 1. iNOS immunoreactivity in control group (a) with weak nuclear immunoreactivity (arrow indicates negative immunostaining) and moderate in muscular fibers were demonstrated. Statin group (b) showed that moderate immunoreactivity in nucleus and strong immunoreactivity in fibers where in the resveratrol+atorvastatin group (c) negative immunostaining in nucleus, weak immunoreactivity in fibers were observed. eNOS showed similar results in control group (d) and in resveratrol+atorvastatin group (f) with weak immunoreactivity in nucleus and weak in fibers. On the other hand, statin group (e) with moderate nuclear immunostaining and weak muscle fiber staining were observed. Arrows showed positive immunoreactivity (×400).

(Olympus BX-51, Olympus, Tokyo, Japan) equipped with a camera (Olympus C-5050 digital camera, Olympus). Image was transferred to a desktop computer system and length of muscle fibrils determined using image analysis software program (Image-Pro Express, Media Cybernetics, Bethesda, MD) for morphometric analysis. Evaluation of the specimen was performed by an experienced histologist blinded to the surgical groups.

Statistical analysis

The statistical significance of differences of groups was analyzed by one-way analysis of variance (ANOVA) or Student's t-test. p-Values of < 0.05 were considered significant.

RESULTS

Observations

After the 10th day of atorvastatin treatment, rats showed piloerection, hunched posture, thin appearance with weight loss, pale appearance and decreased activity. Control group and resveratrol + atorvastatin group showed no significant alterations.

Immunoreactivity

Control group showed weak iNOS immunoreactivity in nucleus and moderate immunoreactivity in muscular fibers. 40 mg/kg atorvastatin for 14 days has resulted with a moderate immunoreactivity in nucleus and strong immunoreactivity in fibers. Co-administration of 20 mg/kg i.p resveratrol for 14 days with atorvastatin elicited a negative immunostaining in nucleus and weak immunoreactivity in fibers (Figure 1a, b and c). eNOS immunoreactivity results were similar in control group and in resveratrol + atorvastatin group, with weak immunoreactivity in nucleus and weak in fibers. On the other hand, in atorvastatin group, moderate nuclear immunostaining and weak muscle fiber staining were observed (Figure 1d, e and f).

Structural modification of rat skeletal muscles

Our results showed that the treatment of rats with atorvastatin have resulted with a significantly shortened muscle fibrils in trapezius, gastrocnemius, semitendinosus and biceps femoris muscles (20.48 ± 0.91, 18.07 ± 1.6, 19.53 ± 1.1, 20.52±1.0 µm, respectively) when compared with control group (34.22 ± 1.07, 32.01 ± 1.52, 33.0 ± 2.10, 31.65 ± 1.0 µm, respectively) ($p < 0.05$ for trapezius, gastrocnemius, semitendinosus and $p < 0.01$ for biceps femoris). Co-treatment with resveratrol prevented the shortening of muscle fibrils caused by statin treatment alone. Fibril lengths were 29.98 ± 2.4, 28.32±1.6, 27.84±1.9, 40.10±0.8 µm in resveratrol + atorvastatin

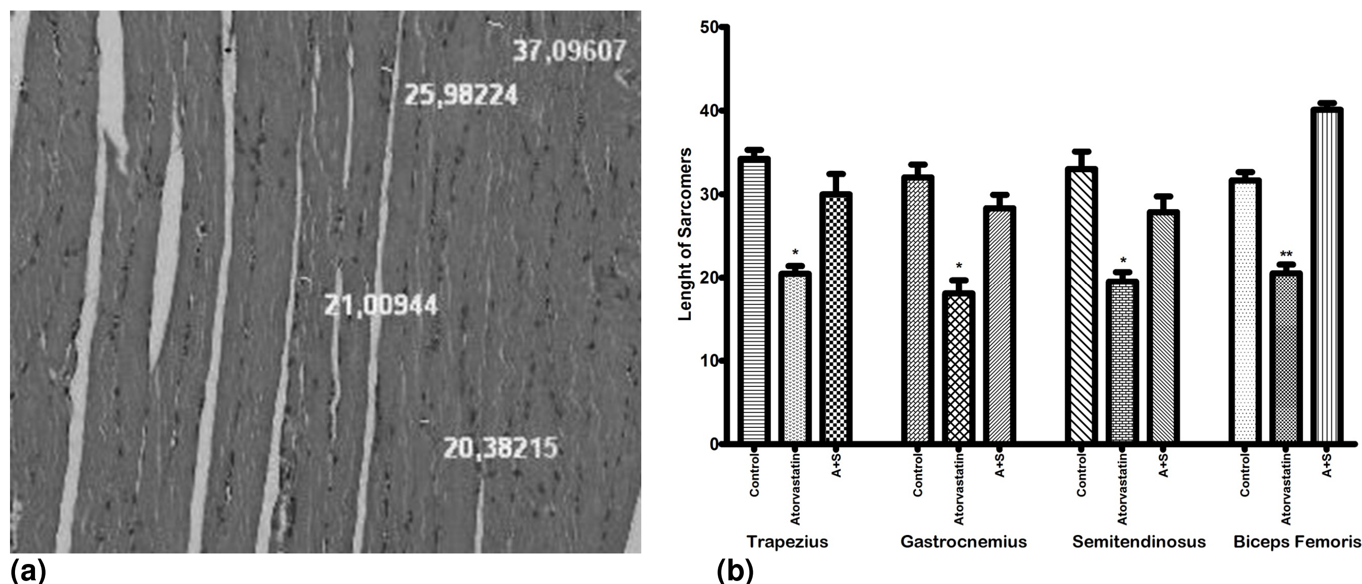

Figure 2. (a) Displays representative skeletal muscle fibril images of rats treated with atorvastatin measured from different fibers. Comprehensive analysis of the length of fibers are shown in (b). As shown in (b), treatment of rats with atorvastatin has significantly shortened fibril length in trapezius, gastrocnemius, semitendinosus and biceps femoris muscles compared with control group. Resveratrol co-treatment prevented the shortening of fibrils caused by atorvastatin treatment alone. Strong correlation between the atorvastatin dose and shortening of fibrils has been evaluated and resveratrol treatment has prevented this effect of statins (*p < 0.01, **p < 0.05). A+S: resveratrol+atorvastatin group

treated groups for trapezius, gastrocnemius, semitendinosus and biceps femoris muscles, respectively (Figure 2a and b). A strong correlation between the atorvastatin and shortening of muscle fibrils has been found, and treatment with resveratrol has prevented the effect of atorvastatin.

DISCUSSION

Indications for statin therapy as recommended by The National Institute for Health and Clinical Excellence were defined as: ischemic heart disease (angina, myocardial infarction, chronic heart disease), cerebrovascular accidents (transient ischemic attack), hypercholesterolemia, peripheral arterial disease and combination of risk factors (hypertension, type 2 diabetes mellitus, age > 70 years). They also recommend that adults with a history of cardiovascular disease (CVD) and adults with a 10-year risk of developing CVD equal to or greater than 20% should start statin therapy as primary prevention (NICE, 2008). These indications make statins one of the world's most prescribed drugs.

Effects of statins on early cellular changes, inflammation markers, have been shown in rats. Our study has also evaluated muscle fiber lengths in early myositis in rats. Atorvastatin treatment has significantly shortened the fibrils of skeletal muscles. Resveratrol has completely reversed the shortening of fibrils, showing its protective effect on atorvastatin induced myositis.

Similar effect of statins has been shown in zebra fish and this effect has been attributed to an inhibition on biosynthetic pathway or an impaired production of mevalonate caused by statins. Such a destructive effect on myosin filament might result with impaired muscular functions which can be projected into clinical symptoms of myopathy (Huang et al., 2011). To our knowledge, this is the first study showing the shortening of myosin fibril in a rat toxicity model for skeletal muscles with deleterious muscle manifestations induced by atorvastatin. Since some muscle biopsies have documented statin-associated myopathy with normal creatine kinase (CK) levels (Phillips et al., 2002); the use of creatine phosphokinase (CPK) as a reliable biomarker for muscle diseases has consequently been questioned (Gunst et al., 1998; Phillips et al., 2002).

CPK levels do not invariably correlate with clinical symptoms of myopathy because elevated CPK values can be associated with various diseases. Myosin fibril length could also be a marker for myositis in patients with normal CK levels. A change of the myosin fibril length associated with myopathy might allow a diagnosis of myopathy before the occurrence of clinical and laboratory symptoms such as elevated CPK levels. All isoforms of

nitric oxide synthase are expressed in skeletal muscle of all mammals (Stamler and Meissner, 2001) and it has been shown that inflammatory cytokines upregulates iNOS in myositis (Williamset al., 1994; Park et al., 1996).

Our results showed that atorvastatin caused an increase in iNOS and eNOS and when resveratrol was co-administered, iNOS and eNOS upregulation has been recovered to control group. This possible down regulation of iNOS in resveratrol + atorvastatin group can be attributed to the effect of resveratrol which has been shown to inhibit iNOS induction in skeletal muscle (Centeno-Baez et al., 2011). Anti-inflammatory effects of resveratrol have been shown in several studies but for the first time, we have shown the effect of resveratrol on iNOS regulation when co-administered with atorvastatin.

Myotoxic effects of atorvastatin are known to be dose-dependent. As pointed out, impaired metabolism of statins, pharmacokinetic interactions and/or genetic effects are all probable causes of the myotoxicity (Laaksonen, 2006). Co-administered agents effecting on similar metabolic pathways (eg fibrates) increases their myotoxic side effects besides their lipid-lowering action (Ballantyne et al., 2003). An alternative molecule that has the same protecting effect by a different mechanism is needed to decrease the adverse effects of atorvastatin without affecting its effects. With further studies, resveratrol could be an alternative to decrease the effects of statins on skeletal muscle.

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Full Length Research Paper

Intravenous levetiracetam of hospitalized patients in Srinagarind Hospital

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Levetiracetam (LEV) is a fairly new antiepileptic drug with broad effectiveness in controlling seizures. Presently, there is limited clinical data worldwide. This study aims to add more clinical efficacy and safety data for intravenous LEV. Chart review was done in patients who received intravenous LEV at Srinagarind Hospital, Khon Kaen University, Thailand from August, 2010 to June, 2012. There were 48 prescriptions on 46 patients with a mean age of 56.75 years. The three most common causes of seizures were metabolic derangement, renal dysfunction, and hypoxic ischemic encephalopathy. Intravenous LEV was used for status epilepticus (SE) for 34 out of 48 prescriptions (70.8%) and non-SE 14 times (29.2%). The loading and maintenance doses of intravenous LEV were 1520.60 mg (range 1000 to 6500) and 1171.70 mg/day (500 to 3000). Seizure was controlled by intravenous LEV in 21 out of 34 prescriptions with SE (61.8%) and all patients with non-SE (100%). The overall mortality rate was 45.7% (21 out of 46 patients). The most common cause of death was sepsis with multiple organ failure (17 out of 21 patients or 81%). There was no obvious side effect of intravenous LEV in any patient. Intravenous LEV is effective and safe in seizure control particularly in patients with renal and liver dysfunction who had either SE or non-SE.

Key words: Levetiracetam, efficacy, safety, status epilepticus.

INTRODUCTION

Epilepsy is one of the most prevalent neurological diseases and a public health problem, because of its chronicity and necessity for continual treatment. The aim of the treatment is to prevent seizures and lessen unwanted side effects from antiepileptic drugs (AEDs). The decision on which AED is appropriate for each patient depends on other factors than just the epileptic type, such as pharmacokinetics, pharmacodynamics, comorbid diseases or concurrent drugs, factors related to each patient including sex, age, and liver and kidney function (Stein and Kanner, 2009).

Levetiracetam (LEV) is from a fairly new group of AEDs with a derivative structure of pyrrolidone and wide antagonizing mechanisms against seizures. LEV binds at a binding site at synaptic vesicle protein 2A (SV2A), and has a restraining effect on secretion of neurotransmitters in the presynaptic area (Lynch et al., 2004; Yang et al., 2007). It inhibits secretion of calcium from neuronal stores and activation of neurons without interfering with normal activation. Additionally, it has been shown that LEV does not involve inhibitory and excitatory neuro-transmission. Approved indications of LEV include:

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(1) use as a mono-therapy for partial onset seizure with or without secondarily generalization in patients of 16 years of age and over who are diagnosed as new epileptic patients, (2) use as an add-on therapy for partial onset seizures in adults and children of 4 years of age and over, (3) the use as an add-on therapy for myoclonic seizures in adults, in teenagers of age 12 and over having juvenile myoclonic seizures, and primary generalized tonic-clonic seizures in both adults and children from 4 years old (Lyseng-Williamson, 2011).

There are 2 types of available LEV forms at Srinagarind Hospital, namely, the 250 or 500 mg pills and the 100 mg/ml intravenous form. A loading dosage of intravenous LEV is 20 to 25 mg/kg and a maintenance dose is 20 to 25 mg/kg by continuous infusion over 24 h. Its properties are different from the conventional AEDs. Less than 10% of LEV binds to protein and does not use the cytochrome P450 or liver glucuronidation, resulting in fewer risks for interaction with other AEDs or other drugs. Prescriptions of LEV have increased, because of the potential use of both oral and intravenous drug administration together with patients' tolerance (Noachtar et al., 2008; Wu et al., 2009; Kwan et al., 2010). This study evaluated the efficacy of intravenous LEV in adult persons with epilepsy (PWE) at Srinagarind Hospital.

METHODOLOGY

The descriptive study is based on retrospective data from medical records of patients of 15 years of age and over who received intravenous LEV at Srinagarind Hospital, Khon Kaen University from August, 2010 to June, 2012. The data consisted of age, sex, seizures, comorbidities, indications, dosage, clinical outcome, and mortality.

Operation definition

Seizure control

Seizure control is defined clinically as no seizure occurrence after intravenous LEV treatment or without additional AED treatment or increasing the dose of LEV. "Uncontrolled" is defined by a seizure occurrence despite intravenous LEV treatment and additional AED intervention, relapse seizures, or requiring an increase of the dose of LEV.

Order of prescriptions of AED

The first line AED is the first drug a patient received to control seizures. If the seizure is uncontrolled, an additional AED is prescribed by attending physicians.

Causes of seizures

Renal dysfunction refers to the case where the patient has blood creatinine levels higher than 1.5 mg/dl. Hepatic dysfunction refers to the case where the patient has over 3 times of values of normal liver function or has evidence of cirrhosis. Metabolic disorder means an abnormality of blood components such as sodium, calcium, urea, or glucose, etc. Encephalopathy indicates post-cardiac arrest patients with hypoxic ischemic encephalopathy diagnosed by computed tomography of the brain (Legriel et al., 2008).

RESULTS

From August, 2010 to June, 2012, there were 48 prescriptions for 46 patients with a mean age of 56.75 years (range 15 to 91 years). Of these, 21 patients (45.7%) were male and 9 patients (19.6%) had a history of epilepsy (Table 1). Computed tomography of the brain was done on 36 patients (78.3%) with abnormal findings 28 times (77.8%). None of the patients had magnetic resonance imaging. Electroencephalograms were done 31 times and had epileptic discharge 20 times (64.5%). The three most common causes of seizures were metabolic derangement, renal dysfunction, and hypoxic ischemic encephalopathy (Table 2).

Intravenous LEV was used for status epilepticus (SE) for 34 out of 48 prescriptions (70.8%) and non-SE 14 times (29.2%). It was used as the first-and second-line AED in 9 (18.8%) and 23 times (47.9%); the other prescriptions were third- to eight-line treatment. The loading and maintenance doses of intravenous LEV were 1520.60 mg (range 1000 to 6500) and 1171.70 mg/day (500 to 3000). One patient who weighed 100 kg received the loading dose of 6500 mg (three times of the usual loading; 2500, 2000, 2000 mg). Seizure was controlled by intravenous LEV in 21 out of 34 prescriptions with SE (61.8%) and all patients with non-SE (100%). The overall mortality rate was 45.7% (21 out of 46 patients), 48.5% in SE group (16 out of 33 patients) and 38.5% in non-SE group (5 out of 13 patients) as shown in Table 3. The most common cause of death was sepsis with multiple organ failures (17 out of 21 patients or 81%). There were no obvious side effects of intravenous LEV in any patient.

DISCUSSION

Since August 2010, intravenous LEV has been used as an AED at Srinagarind Hospital. Presently, clinical data for LEV is limited. There are only 368 reported patients with intravenous LEV treatment, mostly from Europe. This study added to the efficacy and safety data of intravenous LEV on seizure control. Intravenous LEV was effective in non-SE compared to SE patients (100 versus 61.8%). Not surprisingly, seizure control was higher in non-SE patients. The mortality rates in both groups were still high (48.5% in SE group and 38.5% in non-SE group). The efficacy of intravenous LEV varied from 44 to 100% and 95% in seizure control of SE and non-SE patients (Table 4). Misra et al. (2012) reported that 10 out of 38 SE patients who received intravenous LEV died in hospital (26.3%) and 20 more patients were considered

Table 1. Demographic and clinical characteristics.

Characteristic	Number	Percentage
Number of patients	46	-
Men/Women	21/25	43.7/56.3
Number of prescription	48	-
Age (min.-max.) years	56.75 (15-91)	-
Previous history of epilepsy	9	19.6
Co-morbidity	51	-
Seizure type		
Non status epilepticus	14	29.2
Focal seizures	2	-
Complex partial seizures (CPS)	2	-
Generalized tonic-clonic seizures (GTC)	8	-
Myoclonic seizure	2	-
Status epilepticus (SE)	34	70.8
Convulsive SE (CSE)	15	-
Non-convulsive SE (NCSE)	19	-
CT brain	36	75
Abnormal	28	77.8
Normal	8	22.2
EEG		
No epileptic form discharge	6	19.4
Epileptic form discharge	20	64.5
Others	5	10.3

Table 2. Precipitating causes of seizures.

Cause	Number
Metabolic derangement	15
Renal dysfunction	14
Encephalopathy	14
Liver dysfunction	8
Acute ischemic stroke	6
Intracerebral hemorrhage	6
Post cardiac arrest	4
Drug induced seizure	4
Epilepsy	3
Old ischemic stroke	3
Autoimmune disease	3
Brain tumor	2
Thrombotic thrombocytopenia purpura	2
Unknown	2

Patients may have more than one precipitating causes.

possible deaths, because they left the hospital with deterioration. The mortality rate in SE in the Misra study was somewhat lower than in this study. For non-SE patients, the mortality rate in the study was still high. It was assumed that the high mortality rate in this study may be explained by high numbers of comorbidity conditions. All of the patients had at least one pre-existing condition. The overall numbers of comorbidities were 51 (Table 1). Even though the seizure control rate was quite high, the mortality rate was still high. The cause of death was not seizure related but mostly from septicemia and organ failure.

The clinical data on efficacy and order of intravenous LEV in SE are still controversial. Alvarez et al. (2011) reported that intravenous LEV was not as effective as sodium valproate for seizure control in SE after benzodiazepine treatment. In contrast, Gámez-Leyva et al. (2009) showed that intravenous LEV had potential effectiveness for SE treatment. The appropriate order of intravenous LEV in SE treatment is still obscure. In this study, intravenous LEV was used as the additional AED for SE. Almost half of SE patients received intravenous LEV as the third-line treatment.

Most patients (37 patients, 80.43%) had metabolic derangements, primarily renal or hepatic dysfunctions.

Table 3. Seizure stopping outcomes and mortality.

Characteristic	Times (no. of patients)	Outcome		Death (n)
		Seizure controlled, times	Uncontrolled seizures, times	
Status epilepticus	34 (33)	21	13	16
Non-status epilepticus	14 (13)	14	0	5
Total	48	35	13	21

Table 4. Previous reports of intravenous levetiracetam.

Study	Country	Population	Outcome (terminated seizure %)
Retrospective author			
Ruegg (2008)	Switzerland	50 (SE: 24, non SE: 19, Prophyl axis:7)	SE: 16/24 (67%), non SE: 18/19 (95)
Knake (2008)	Germany	18 SE	18/18 (100)
Moddel 2009	Germany	36 SE	25/36 (69)
Gamez-Leyva (2009)	Spain	34 SE	24/35 (71)
Berning (2009)	Germany	32 SE	30/32 (94)
Fattouch (2010)	Italy	9 SE	7/9 (78)
Aiguabella (2011)	Spain	40 SE	23/40 (58)
Alvarez 2011	Switzerland	58SE	30/58 (52)
Prospective author			
Misra (2011)	India	48 SE	36/48 (75)
Eue (2009)	Germany	43 SE	19/43 (44)

LEV is indicated and safer than other AEDs for these patients, because it does not have interaction with the cytochrome P450 isoenzyme (Lyseng-Williamson, 2011). There were four patients in the present series that were antibiotic associated seizures (3 patients received ceftazidime and 1 patient received cefazolin). All patients developed non-SE seizures, had renal dysfunction, and were controlled by intravenous LEV (Ozturk et al., 2009). Intravenous LEV may be an appropriate AED for this particular type of seizure.

The mechanism of action of LEV to control seizures is unknown. The possible mechanisms are the inhibition of voltage-dependent N-type calcium channels, facilitation of GABA-ergic inhibitory transmission through displacement of negative modulators, reduction of delayed rectifier potassium current, and/or binding to synaptic proteins which modulate neurotransmitter release. Intravenous LEV use may be increased in the future. There are some reported side effects including Steven Johnson syndrome, bullous pemphigoid, and aggravation of myoclonus and non-convulsive SE (Isoda et al., 2012; Karadag et al., 2012; Liu et al., 2012; Zou et al., 2012). Rare conditions such as hypersexuality are also reported after LEV use (Metin et al., 2012). Even though in this study, there were no serious complications of intravenous LEV. Side effects of LEV are still needed to be monitored.

Conclusion

Intravenous LEV is effective and safe in seizure control particularly in patients with renal and liver dysfunction who had either SE or non-SE seizures.

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Full Length Research Paper

Pharmacokinetics of isoniazid in Ethiopian children with tuberculosis in relation to the N-acetyltransferase 2 (*NAT2*) genotype

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Until recently, the dose of isoniazid used to treat tuberculosis was the same for all patient groups. However, the pharmacokinetic profile of isoniazid varies across different populations. A comparative, observational, single-dose, 5 h study was conducted evaluating the pharmacokinetics of isoniazid in Ethiopian children. Pharmacokinetic parameters for a dose of 5 mg/kg and NAT2 genotype were determined in 29 children with tuberculosis (<15 years, with mean age of 8.6). Initially, univariate analyses evaluated covariates that exhibited associations ($p < 0.2$) with isoniazid pharmacokinetic parameters. Covariates with associations, acetylator genotype ($p < 0.01$) and age ($p < 0.1$) were further analysed with multiple linear regression. Sixteen (55%) were genotyped as rapid and 13 (45%) as slow acetylators. Four rapid acetylators had 2 and 3 h post-dose concentrations of < 3 and 1.5 ug/ml, respectively. Multiple linear regression analyses revealed acetylator status to be the only predictor of k, area under the curve ($AUC_{2\rightarrow 5h}$), and isoniazid concentrations at 2, 3, 4 and 5 h. The mean values of these variables were also found to differ between genotypes ($p < 0.0025$). These findings reaffirm that 5 mg/kg isoniazid dose may not provide adequate plasma drug levels in all paediatric patients. Thus, isoniazid dose for children should be higher than 5 mg/kg body weight to cover the diverse acetylation kinetics.

Key words: Acetylator status, paediatric tuberculosis, isoniazid, pharmacokinetics, NAT2, Ethiopia.

INTRODUCTION

Six decades after its introduction into clinical practice, isoniazid (INH) remains a main-stay agent of all first-line anti-tuberculosis (anti-TB) regimens owing to its remarkable bactericidal activity against the metabolically active organisms and relatively low toxicity profile. INH is also a valuable component of anti-TB regimens as it prevents the emergence of resistant strains against companion agents (Donald et al., 2004, 2007; Mitchison 2000; Vilchèze and Jacobs, 2000). The pharmacokinetics of INH in humans has been well characterized. After both

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oral and parenteral administrations, it is rapidly and completely absorbed and peak plasma concentrations usually occur within 1 to 2 h after oral administration (Weber and Hein, 1979). INH exhibits poor plasma protein binding pattern, and crosses biological membranes readily with appreciable distribution into body compartments (Schaaf et al., 2005). The main metabolic route of INH is acetylation to acetyl-INH by arylamine N-acetyltransferase-2 (*NAT2*) enzyme (Donald et al., 2007).

The common genetic polymorphism responsible for the pharmacogenetic variation as rapid and slow acetylator in INH metabolism involves the *NAT2* gene, and response of the different genotypes to INH differs considerably (Donald et al., 2007). It has been shown that *NAT2* genotype accounts for considerable variability in INH pharmacokinetics (Chen et al., 2009; Gumbo et al., 2007; Schaaf et al., 2005).

The pharmacokinetic profile of INH varies in different patient populations. This has been extensively studied in adults. However, the kinetic data for children particularly that embrace the acetylator genotype are scanty, and the available evidence is derived from few studies (McIlleron et al., 2009; Schaaf et al., 2005).

The one size fits all INH dosing approach, where both adults and children prescribed INH doses of 4 to 6 mg/kg/day for the treatment of tuberculosis was adopted for decades by many professional bodies including the World Health Organization (WHO) (2003). In recent years, however, there are some amendments in the recommendations that children should receive higher INH doses than adults. For instance, WHO (2010) and the South African national guidelines (2004 and 2009) have now changed the INH dose recommendations from 5 to 10 mg/kg, with a range of 10 to 15 mg/kg. On the other hand, National programs such as in Ethiopia (2010) still follow the classic one size INH dosing approach.

In selecting the optimum dosage regimen, extrapolation of the findings from adult studies to children would be irrational. The pharmacokinetic profile of the drug in children who are efficient metabolisers could significantly differ from that in adults. This study therefore attempted to assess the pharmacokinetic aspects of INH in children prescribed 5 mg/kg INH dose for treatment of tuberculosis (TB) in Ethiopia.

MATERIALS AND METHODS

Study design

The study was undertaken in Paediatric ward of Tikur Anbessa teaching hospital in Addis Ababa, Ethiopia. Children between 1 to 15 years of age admitted for treatment of newly diagnosed TB (all forms) were recruited after written informed consent obtained from parents/guardians. Verbal assent was also obtained for children above age of 12 years. Patients were excluded if they had already been on an anti-TB regimen, were severely ill, had clinical evidence of liver damage or anaemia, showed sustained dissent or if consent was not obtained. An earlier study by Schaaf et al. (2005) has indicated the average mean values for 2 h post dose plasma concentrations of INH in rapid (fast and intermediate) and slow acetylator children to be 4.5 and 8.6 µg/ml, with average standard deviations of 1.974 and 1.81, respectively. Win Episcope software adopted from Epi Info was used to compute the sample size using means and standard deviations from the above study. Accordingly, a sample size of 14 (7 rapid and 7 slow acetylators) was calculated to assess the effect of acetylator genotype on INH pharmacokinetic variables, with a confidence interval of 95%. The effect size was 2.1, and to detect this effect size a power of 90% was assumed. The actual sample size was increased until the required number of the study subjects in both categories was attained.

A standard INH powder obtained from Sigma-Aldrich Inc. was used for pharmacokinetic analysis. INH was administered orally at a nationally prescribed dose of 5 mg/kg body weight to overnight fasted treatment-naïve children. Blood specimens of 1.5 to 2 ml each were collected at 2, 3, 4, and 5 h after dosing and were centrifuged (3000 *g* × 3 min) at 0°C immediately after each collection to separate the plasma samples. The plasma samples were then stored at -80°C until analysis. The multi-drug anti-TB regimen was initiated on the day following the 5 h INH administration. A 2 to 3 ml blood sample was further taken for genotyping assay on the sixth day after the first sample collection and was stored at -20°C.

Plasma INH concentration determination

INH concentrations were quantified by established high performance liquid chromatography (HPLC) method (Seifart et al., 1995). The accuracy of the HPLC method in terms of average percentage recovery was determined after five repeated injections at nine appropriately spaced calibration points and was found to be 98.8% (concentration value (CV) ± 2.2%), with a percent recovery of 96.7% (CV ±3.1%) at lower limit of quantification (0.5 µg/ml). The intra-sample coefficient of variations of the concentration values were also less than 5%.

NAT2 genotype determination

Genomic DNA (gDNA) was extracted from the whole blood by using the ArchivePure™ DNA Purification Kit (5 PRIME Inc.), according to the manufacturer's protocol. A DNA fragment spanning 866 bp, which contained the whole coding region of the NAT2 gene, was amplified from gDNA by polymerase chain reaction (PCR) with two previously reported primers (O'Neil et al., 2000) 5'-GGCTATAAGAACTCTAGGAAC-3' and 5'-AAGGGTTTATTTGTTCCTTATTCTAAAT-3'. A combination of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele specific PCR was applied for analyzing NAT2*4, NAT2*5, NAT2*6, NAT2*7, NAT2*14, and NAT2*17 alleles on the basis of previously described methods (Cascorbi et al., 1995) with some modifications. Subjects that are either homozygous or heterozygous to the wild NAT2*4 allele were categorized as rapid acetylators, whereas those with homozygous or two heterozygous mutant alleles were taken as slow acetylators.

Pharmacokinetic parameters

The AUCs over the interval 2 to 5 h ($AUC_{2\rightarrow5h}$) were calculated from INH concentration-time plot by trapezoidal rule. The apparent first

Table 1. Clinical and demographic features of children with tuberculosis assessed for acetylation status.

Clinical or demographic features	Number	%
Age group (years)		
≤ 4	6	20.7
4-9	8	27.6
9-15	15	51.7
Sex		
Male	16	55.2
Female	13	44.8
Body Mass Index		
< 18.5	15	51.7
18.5-25	14	48.3
Type of TB		
Pulmonary	14	48.3
Extrapulmonary	8	27.6
Disseminated	7	24.1
Extrapulmonary TB		
Peripheral TB lymphadenitis	6	75
TB pericarditis	1	12.5
TB spondylitis	1	12.5
Concomitant drug use		
Yes	5	17.2
No	24	82.8
Rapid acetylators alleles		
4*/4*	2	6.9
4*/5*	6	20.7
4*/6*	7	24.1
4*/14*	1	3.5
Slow acetylators alleles		
5*/5*	3	10.3
5*/6*	8	27.6
5*/7*	1	3.5
6*/7*	1	3.5

order elimination rate constant (k, h^{-1}) of the individual patients were obtained from slope of linear regression of the natural logarithm of INH concentration at time t (lnCt) and t from 2 to 5 h.

Statistical analyses

The INH pharmacokinetic data were summarized as mean value of the groups with respective standard error of the mean. The covariate effects of age, weight, sex, acetylator genotype, type of TB and concomitant drug use on the pharmacokinetic variables (k, $AUC_{2\rightarrow5h}$, and 2 to 5 h INH concentrations) was assessed by linear regression analyses. A logarithmic transformation was used on k values to reduce skewness and to improve the distribution of residuals. A multiple linear regression was further performed to evaluate the effects of covariates that were found to be possibly associated ($p < 0.2$) with INH pharmacokinetics in the univariate analyses or that have literature support. Colinearity diagnostics were employed to rule out possible multicolinearity between variables. Student t-test was used for unpaired data to test statistically significant group mean differences. Levene's test was also used to assess the homogeneity of group variance prior to a t-test parametric procedure. The relation between age and pharmacokinetic parameters were reassessed across the stratified genotypes by linear regression. Significance was set at $p < 0.05$. The statistical analyses were performed with statistical package for social sciences (SPSS), version 15.

Ethical considerations

The study protocol was reviewed and approved by the Armauer Hansen Research Institute/All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre (AHRI/ALERT) and National Research Ethics Review Committees.

RESULTS

Patient characteristics

A total of 29 newly diagnosed inpatient paediatric TB patients with a mean age of 8.7 years (1.5 to 14.8 years) were enrolled in the study. Table 1 summarizes details of demographic and clinical features of the participants.

Genotyping

The presence of single nucleotide polymorphisms (SNPs) at positions 191, 341, 434, 590, and 857 of *NAT2* gene was tested for all 29 patients. Among the SNPs tested, NAT2*5 (341T > C) was the most frequently seen SNP with occurrence rate of 36.2%, followed by NAT2*6 (590G > A) which occurred in 27.6% of the cases. The least frequent SNPs were NAT2*7 (857G > A) and NAT2*14 (191G > A), with frequencies 3.5 and 1.7%, respectively. The wild type NAT2*4 that lacks any of these SNPs was observed with an occurrence rate of 31%. The genotype data also showed that *5/*6 was the most frequent haplotype, which occurred in 8 (28%) of the patients followed by *4/*6 and *4/*5 with 7 (24%) and 6 (21%) occurrence rates, respectively. Accordingly, 16 children (55%) with a median age of 9 years were genotyped as rapid acetylators, and 13 (45%) with a median age of 11 years as slow acetylators. Among the rapid acetylators, only 2 (13%) were homozygous (fast) and the rest 14 (87%) were heterozygous (intermediate)

Table 2. Degree of associations obtained between INH pharmacokinetic variables and acetylator status, age and weight from multiple linear regression analyses.

Variables	2 h INH conc. [β±SE* (Sr²**)]	3 h INH conc. [β±SE * (Sr²**)]	4 h INH conc. [β±SE * (Sr²**)]	5 h INH conc. [β±SE * (Sr²**)]	AUC2-5 h [β ±SE* (Sr²**)]	K (log) [β ±SE* (Sr²**)]
Slow acetylator status	1.50±0.37ᵃ (0.349)	1.31±0.33ᵃ (0.31)	1.10±0.26ᵃ (0.336)	0.91±0.24ᵃ (0.291)	3.74±0.91ᵃ (0.341)	-0.15±0.05ᵃ (0.262)
Age (years)	0.65±0.10 (0.009)	0.14±0.09 (0.053)	0.13±0.07 (0.066)	0.12±0.07 (0.065)	0.37±0.25 (0.044)	-0.02±0.01 (0. 046)
Weight (kg)	0.07±0.04 (0.001)	0.02±0.03 (0.012)	0. 04±0.03 (0.029)	0.03±0.03 (0.029)	0.08±0.10 (0.014)	-0.01±0.01 (0.057)

*Regression coefficients ± Standard error ** Semipartial correlations [a] p < 0.01.

acetylators.

Pharmacokinetics

HPLC chromatograms with well resolved peaks were obtained for all samples. INH concentration at 2 h after dosing, which is frequently quoted in the literature as a convenient reference point, varied from as low as 1.76 µg/ml to as high as 7.67 µg/ml (Figure 1). A 2 and 3 h post-dose INH plasma concentration of 3 to 5 µg/ml (Peloquin et al., 1996) and 1.5 µg/ml (Schaaf et al., 2005), respectively have been suggested as a required range for optimal bactericidal effects. Even though the above validated therapeutic ranges for INH were achieved in majority (86%) of the study subjects, four (14%) had 2 and 3 h concentrations less than 3 and 1.5 µg/ml, respectively. Among these four, one even failed to achieve a concentration of 2 µg/ml at 2 h.

Univariate regression analyses showed that there was no association ($p > 0.2$) between INH pharmacokinetic parameters and the covariates: weight, sex, type of TB and concomitant drug use. Acetylator genotype ($p < 0.01$) and age ($p < 0.1$), were however found to be strongly and weakly associated, respectively with the kinetic parameters. Since several pharmacokinetic studies in children strongly support the relationship between weight and drug elimination (Serrano et al., 1999; Trenque et al., 2004; Laer et al., 2005; Aumente et al., 2006; El-Tahtawy et al., 2006; Anderson and Holford, 2008), the effect of weight on INH pharmacokinetics was reassessed with acetylator genotype and age using multiple regression analysis. The analyses revealed that only acetylator status was found to be a strong predictor of all pharma-cokinetic parameters (k, $AUC_{2\rightarrow 5h}$, and 2 to 5 h INH concentrations), while age and weight failed to make any contribution to the model (Table 2). For this model, the correlation coefficient (R) was significantly different from zero ($p < 0.01$) for all set of variables. The adjusted R^2 values indicate that around 40% of the variability in INH concentrations could be explained by the model. Even though there appeared to be a considerable overlap in the observed variables between the geno-types, plasma INH concentrations and $AUC_{2\rightarrow 5h}$ were higher and elimination rate constants lower in the slow compared to the rapid acetylators. For instance, the lowest 2 h INH concentration (1.76 µg/ml) was observed in a rapid acetylator subject, and the highest (7.67 µg/ml) was noted in a slow acetylator. Comparison of the 2 h plasma INH concentrations for the two genotypes is illustrated in Figure 1. The homogeneity of group means were tested for each pharmacokinetic variable with 95% confidence, and there was a statistically significant difference in each of the variables between the genotypes ($p < 0.01$ in each instance) (Table 3). In addition, all four observed INH plasma levels below the target ranges occurred in rapid acetylators, with a rate of 25%, and the two homozygous rapid acetylators were amongst them. The proportions of male and female were 9 (56%) and 7 (44%) for rapid acetylators and 7 (54%) and 6 (46%) for slow acetylators, respectively. Thus, tests of homogeneity of group means of pharmacokinetic variables of the two sexes were performed within the genotypes by using a two tailed t-test, with 95% confidence. The values of k, $AUC_{2\rightarrow 5h}$, and 2, 3, 4, and 5 h INH concentrations were not significantly different between males and females in both genotypes.

The effect of age on INH pharmacokinetics was further examined by linear regression analysis after stratifying the study subjects into acetylator genotypes. In both genotypes, fitting straight line regressions on age for k, $AUC_{2\rightarrow 5h}$ and INH concentration at the time points of 2, 3, 4, and 5 h was performed. In slow acetylators, with increasing age, a significant decline in transformed k rest of the pharmacokinetic variables was observed. At 95% confidence interval, the deviation

Table 3. The mean first order elimination rate constant (k), the area under the curve (AUC), and the mean plasma INH concentrations at 2, 3, 4, and 5 h after dosing with 5 mg/kg INH in rapid and slow genotypes among children with tuberculosis assessed for acetylation status.

Pharmacokinetic variable		Genotype (n)		p*
		Rapid (16)	Slow (13)	
K (h^{-1})		0.51±0.04	0.34±0.02	0.0024
$AUC_{2\rightarrow5}$ (µg × h/ml)		5.53±0.53	9.62±0.76	<0.0001
INH concentration (µg/ml)	2 h	3.46±0.21	5.03±0.31	0.0002
	3 h	2.12±0.20	3.57±0.28	0.0002
	4 h	1.35±0.15	2.57±0.22	<0.0001
	5 h	0.87±0.13	2.05±0.28	0.0003

Data are mean ± standard error of the mean, n is number of study subjects; k- elimination rate constant; $AUC_{2\rightarrow5h}$ -area under the plasma concentration curve over the interval 2 to 5 h; *p values by student two-tailed t-test to evaluate equality of the pharmacokinetic variables between the slow and rapid acetylator groups.

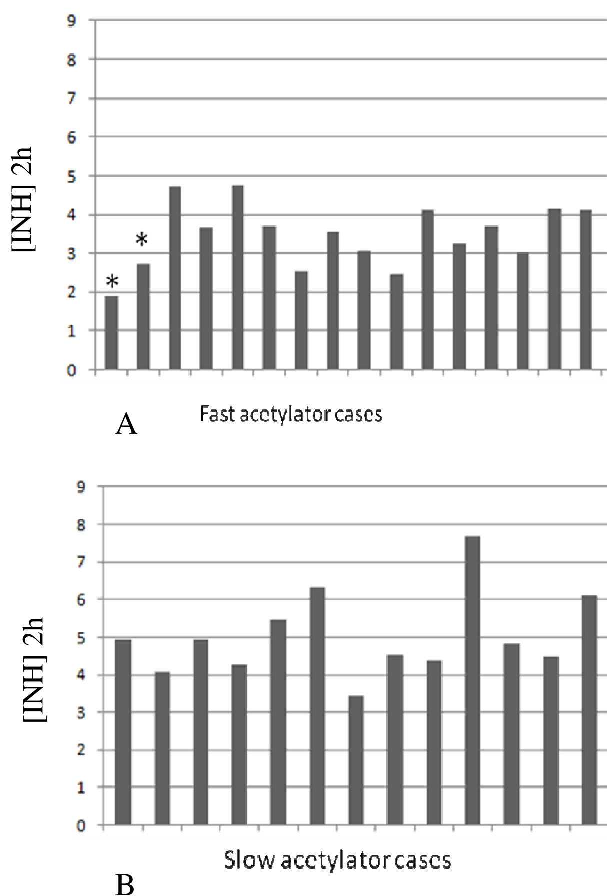

Figure 1: Bar charts showing 2 h Plasma INH concentrations (µg/ml). Slow (A) and rapid (B) acetylators in 29 children with TB after an INH dose of 5 mg/kg. [INH]-isoniazid concentration, *homozygous rapid acetylators

of slope from zero was statistically significant ($p < 0.05$ in each instance). However, the relations obtained in rapid acetylators were neither uniform nor were the slopes significantly different from zero.

DISCUSSION

The influence of acetylator genotype and age on INH pharmacokinetics of children has been well described (McIlleron et al., 2009; Schaaf et al., 2005). In this study, the INH pharmacokinetic profile of the study subjects was best predicted by acetylator genotype, while age contributed to a lesser extent only in the univariate model.

Furthermore, statistically significant differences in INH handling were observed between slow and rapid acetylator children. These results underscore the considerable differences in pharmacokinetic variables of INH between fast and slow acetylator children as reported elsewhere (McIlleron et al., 2009; Rey et al., 2001; Schaaf et al., 2005). Despite the statistically significant differences, a clear margin of drug exposure was not observed among some individuals between the genotypes. Such an overlap between the fast and slow acetylator children was also suggested by Cranswick and Mulholland (2005) in their commentary article on the report of Schaaf et al. (2005). In this study, 25% of the rapid acetylator children did not reach a 2 h concentration of 3 µg/ml and a 3 h concentration of 1.5 µg/ml. The incidence of poor INH plasma levels was also higher in the homozygous than the heterozygous rapid acetylators. These findings, in conjunction with earlier published reports in children (McIlleron et al., 2009; Schaaf et al., 2005) point to the fact that rapid acetylator children would fail to achieve the

desirable concentrations with 5 mg/kg dose of INH, homozygous types being at a greater risk than the heterozygous ones. It is worth noting that the proportion of rapid acetylators that did not achieve the recommended plasma concentration varies with the dose of INH used. For instance, all homozygous rapid acetylators failed to achieve the desired concentration of INH in the present study as well as in the study published by McIlleron et al. (2009).

The proportion of homozygous rapid acetylators who did not achieve the desired concentration came down with increasing INH dose (McIlleron et al., 2009; Schaaf et al., 2005). A different pattern emerges with heterozygous rapid acetylators. The proportion that failed to achieve the desired concentration with 5 mg/kg dose of INH was 14% (present study) and 90% (McIlleron et al., 2009). However, all recruited heterozygous children had achieved the recommended concentration with increasing dose (McIlleron et al., 2009).

Moreover, earlier pharmacokinetic studies conducted in Ethiopian children also showed that drug levels within therapeutic ranges could be achieved with INH dose of 10 mg/kg (Eriksson et al., 1988). These findings reaffirm the notion that higher dosing of INH could diminish the incidence of low drug levels in fast acetylators. In addition, one of the currently recommended doses in children (10 mg/kg) is reported to sufficiently treat majority of the children and is not associated with unacceptable adverse reactions (Cranswick and Mulholland, 2005). Even though the data generated so far provide justification, at least in part, for the recommendation of higher mg/kg doses of INH in children, further studies are required to tailor the higher doses based on disease severity, age and acetylation status for optimal treatment outcomes, with the least possible risk of toxicity.

The study demonstrated that age contributed to the univariate pharmacokinetic model of the slow acetylator group, but not to that of rapid acetylators. The absence of the expected effects of age could in part be explained by strongly skewed age pattern of the study participants (with median age of 9) to the maturation point of NAT2, which is around 10 to 12 years (Pariente-Khayat et al., 1991). Another reason could be the smaller number of younger children within a genotype, as the sample size was not determined, taking into account age influences, which is a limitation of the current study. As age is more essential for defining pharmacokinetics in young children and infants compared with older children (Holford, 2010), further evaluation of INH pharmacokinetics in small children is necessary.

An interesting observation in this study was the low frequency of the NAT2*14 allele. This SNP is considered as a specific feature for black Africans (Hein et al., 2000; Osborne 2003). Surprisingly, this SNP was observed in only one of the 29 Ethiopian study participants. The relative scarcity of this allele in the Ethiopian population was also reported by Yimer et al. (2006), where none of the 128 Ethiopian adults screened for the NAT2*14 allele expressed this SNP. Even though there is not complete absence of the allele in the population, as demonstrated by its presence in one of the study subjects, it nevertheless indicates that the prevalence rate of the NAT2*14 is very low, at least in the studied Ethiopian population.

Conclusion

Our study presents evidence that 5 mg/kg INH dose might not provide adequate plasma levels in paediatric TB patients who are rapid acetylators. Thus, in settings where dosage cannot be tailored based on acetylator status, children should receive a higher mg/kg body weight INH dose to ensure that the rapid acetylators, especially the homozygous, would attain an optimal INH plasma concentration.

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Full Length Research Paper

Methylphenidate effects on search strategy of an animal model of attention-deficit/ hyperactivity disorder

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This study is aimed at investigating the effects of methylphenidate about search strategy use on the spontaneously hypertensive rat, an animal model of attention-deficit/hyperactivity disorder in the Morris water maze. The methylphenidate group rats were given intraperitoneal injection of methylphenidate (10 mg/kg) dissolved in saline 30 min before daily training for 6 consecutive days. In the same way, the model group and the control group rats were only administered equalized volume of saline, respectively. From the second day of trials, the frequency of using tendency-straight strategy to search the hidden platform was significantly increased in all of the training rats. Additionally, the methylphenidate group rats used straight strategy more frequently than the model group rats did on days 1, 3 and 6, respectively. The data indicated that methylphenidate might play a positive role in improving spatial learning ability of the spontaneously hypertensive rat by adding its straight search strategy use in the Morris water maze.

Key words: Methylphenidate, spontaneously hypertensive rat, Wistar Kyoto rat, behaviour, search strategy.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a clinically heterogeneous neuropsychiatric syndrome. Its symptoms such as inattention, hyperactivity, impulsivity and learning deficit cause impairment to academic performances and intellectual function of children (Faraone et al., 2003), and cognitive impulsiveness often makes them to show forgetfulness (Sagvolden, 2000).

With stimulant treatment for children starting in the 1960s, ADHD have been treated with medications such as methylphenidate and so on for over half a century, which serve to alleviate symptoms and to improve ADHD children performances in school (Solanto, 1998). Today, methylphenidate is still widely used in developed and developing countries (Scheffler et al., 2007) despite the emergence of new non-stimulant drugs; however, the effects of methylphenidate on behaviour and learning are not yet fully understood. Search strategies have been used to analyze the performance of rats in the Morris water maze (MWM), which is a useful tool to assess learning and memory ability in rodent models (Vorhees and Williams, 2006). Based on rats' trace images, strategies are often grouped into categories including spatial strategies, repetitive looping strategies and non-spatial strategies, and spatial strategies are considered to be the most efficient method of finding the hidden platform in the MWM (Brody and Holtzman, 2006; Janus, 2004). Additionally, another search strategy classification includes straight, tendency, marginal and random strategies (Jiang et al., 2004).

Although some water maze research has been done on the spontaneously hypertensive (SH) rat (Prediger et al., 2005; Clements and Wainwright, 2006; Pamplona et al., 2009), a genetic animal model for ADHD, because of the similar behavioural problems it shows and deficits in learning and memory as well (Davids et al., 2003; Meneses et al., 2011; Sagvolden, 2000); little is known about the

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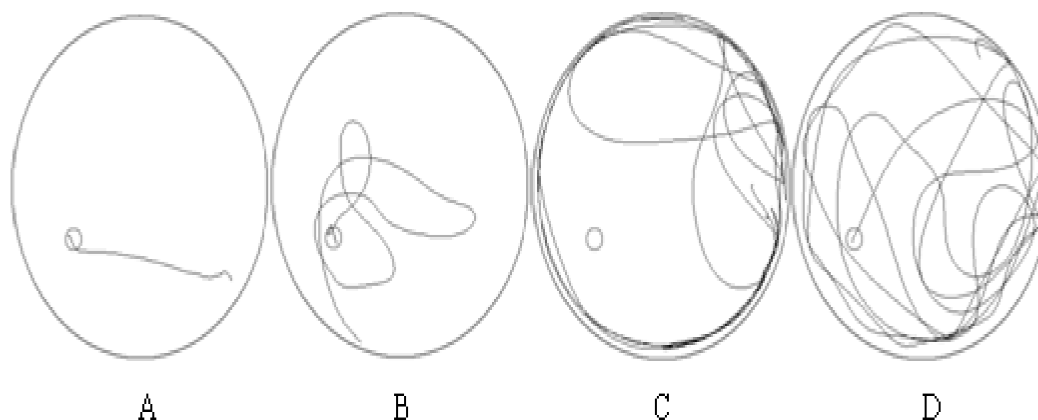

Figure 1. Search strategies used by rats in locating the hidden platform in the Morris water maze. Representative traces of male spontaneously hypertensive rats and Wistar Kyoto rats are shown for (A) Straight strategy, (B) Tendency strategy, (C) Marginal strategy, and (D) Random strategy in the Morris water maze training.

about the effect of methylphenidate on strategy. Therefore, the present study is designed to focus on the effect of methylphenidate on the SH rat's search strategy in place navigation test of MWM.

MATERIALS AND METHODS

Animals

Male SH rats (n=16) and their controls: Wistar Kyoto (WKY) rats (n=8) (Okamoto and Aoki, 1963) of 7-week-old (Slac Laboratory Animal Co. Ltd., Shanghai, China), were used for this experiment. All the rats were kept four per cage (measuring 480×355×195 mm) for 6 days acclimation in an experimental room with a constant relative humidity (50±5%) and ambient temperature (23 ± 1°C). They were maintained on a 12:12 h light: dark cycle (7:00 a.m. to 7:00 p.m.), and had free access to food and water.

Apparatus

The maze consisted of a cylindrical, black painted pool, 130 cm in diameter and 60 cm deep. The apparatus was full of water to a depth of 25 cm, with a temperature of 23 ± 1°C. A black circular escape platform, 10 cm in diameter, was located 1 cm below the water surface, placed in one of four equal-size quadrants, 40 cm away from the wall of the pool. The position of the pool and the extra maze cues were maintained unchanged throughout the study. The position of the rat in the pool was automatically recorded by a video tracking/computer digitizing system (Sunny Instruments Co. Ltd., Beijing, China).

Study design

The SH rats were randomly divided into two groups (each n=8): a model group (MG) and a methylphenidate hydrochloride group (MPDG). The MPDG rats were given intraperitoneal injection of methylphenidate (10 mg/kg, First Suzhou Pharmaceutical Co. Ltd., Suzhou, China) dissolved in saline (0.9% NaCl) 30 min before daily training for 6 consecutive days. The WKY rats were used as a control group (CG). In the same way, the MG and CG rats were only administered equalized volume saline, respectively. All procedures were performed in accordance with Xi'an Jiaotong University guidelines for animal research.

All the rats were allowed to swim 120 s in the pool containing no platform for habituation one day before the experiment. In the following 6 days, the rats were trained to find the hidden platform, which was the only escape from the water. They were given four trials on each day with 15 min intertrial intervals. In each trial, the rat was placed gently in the water facing the pool wall at one of the four starting points (in a random order). If the rat failed to locate the escape platform within 120 s, the experimenter would guide it to the platform where they were allowed to stay for 10 s.

According to swimming traces of rats' locating the hidden platform, the following four search strategies were recorded, including straight (Figure 1A), tendency (Figure 1B), marginal (Figure 1C) and random (Figure 1D). Draw an imaginary straight line from the point the rat was placed into the water to the midpoint of the platform and treat it as the maze's axis. If the distance of each point of a rat's trace from the axis does not exceed 25% of the diameter of the maze, it indicates that the rat formed reference cognition according to the outside fixed clues and the straight strategy which the rat used is considered to be the most efficient method of finding the platform. Essentially different from the random strategy and the marginal strategy, the tendency strategy is similar to the straight strategy; however, the scope of the trace is within 55% of the diameter of the maze. With using the tendency strategy, a rat will not be able to accurately determine the location of the target, but it knows the direction of it. The marginal strategy reflects instinctive behaviour and the tracks of rats usually appear in the edge of the pool; and the random strategy, which is thought to be a blind movement, is used by rats that presented themselves in the most areas of the maze (Jiang et al., 2004).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) 13.0 statistical software (Spss Inc., Chicago, IL, USA). The differences of the search strategies used by the rats among the groups were determined by Mann-Whitney non-parametric test. Significance was assumed at $P<0.05$, 0.01 or 0.001.

RESULTS

Search strategies findings

With the experiment carried out, each group using the straight or the tendency strategy was gradually increasing in percentage. From the beginning to the end of the training, the MPDG rats decreased their use of the random strategy from 56.3 to 0%, increased their use of the tendency strategy from 28.1 to 50% and the straight strategy from 15.6 to 50% (Figure 2B). During the training of the MG, the random strategy use was decreased from 62.5 to 6.2%, meanwhile, the tendency strategy use was increased from 31.2 to 68.8%, and the straight strategy use was also increased from 3.1 to 25% (Figure 2A). As for the CG rats, they decreased their use of the random strategy from 56.2 to 6.2%, increased their use of the tendency strategy from 25 to 71.9% and the straight strategy from 3.1 to 21.9%. The marginal search strategy was mainly seen in the 1st day training of the WKY rats (Figure 2C).

Straight strategy findings

Overall, we could see that the MG rats used the straight strategy with a higher percent as compared to the CG ($P=0.006<0.01$); meanwhile, the MPDG rats used the same strategy with a higher percent as compared to the MG rats ($P<0.001$) during the whole training. Specifically, the MPDG rats used the straight strategy more frequently than the MG rats did on day 1 ($P=0.002<0.01$), day 3 ($P=0.002<0.01$) and day 6 ($P<0.001$), respectively, however, daily comparisons were not significant between the MG and the CG.

Effective strategy findings

Since the straight strategy and the tendency strategy are similar but apparently different from the rest of the two strategies, they were regarded as the effective strategy as compared to the non-effective strategy which included the marginal strategy and the random strategy for further analysis in this study. The results showed that the tendency-straight strategy used from days 2 to 6 was significantly increased as compared to that used on day 1 in the MG ($P_{day2}=0.047<0.05$; $P_{day3,4,5,6}<0.001$, respectively), the MPDG ($P_{day2}=0.001<0.01$; $P_{day3}=0.001<0.01$; $P_{day4,5,6}<0.001$, respectively) and the CG ($P_{day2}=0.024<0.05$; $P_{day3}=0.001<0.01$; $P_{day4,5,6}<0.001$, respectively), respectively (Figure 3).

DISCUSSION

As a first-line drug for ADHD, methylphenidate has been clinically effective in treating the symptoms of the disorder for decades, which are believed to enhance neurotransmission of dopamine and norepinephrine (Spencer, 2004). However, the effects of methylphenidate on behaviour and learning are far from clear. In the MWM hidden platform training, animals can not directly see the platform, so they must perform a search for it until they find the platform to escape from the maze. Accordingly, the SH rat, a validated rat model of ADHD, was used to make investigations on the search strategy in the MWM in the present study.

In view of previous behavioural studies of learning and memory including testing in the water maze, SH rats exhibited a better, poorer or similar performance relative to WKY rats (Ferguson and Cada, 2004; Gattu et al., 1997; King et al., 2000; Wyss et al., 2000). In this study, the data showed that the MG rats and the CG rats had a significant difference in percentage on straight strategy use. Overall, the SH rats seemed better than the WKY rats on accurate target location, but their daily comparisons were not of statistical significance. Although, some WKY rats tends to choose the marginal strategy in the beginning of the training, using tendency-straight strategy was also gradually increasing in frequency over 6 days of trials as well as the SH rats. The two male rat strains showed their improved search ability respectively with the experiment carried out, suggesting a more efficient approach to find the target.

It is well known that the MWM is mainly used to evaluate the hippocampal cognitive function in rodents. A recent study investigated the effects of methylphenidate on cell proliferation and neuronal differentiation in the hippocampus. Among 2.5, 5 and 10 mg/kg, they observed that the numbers of neurons were significantly increased only in the 10 mg/kg methylphenidate group as compared to the vehicle group (Lee et al., 2012). In this study, it was also found out that the SH rats treated with 10 mg/kg methylphenidate chose less random strategy and more straight strategy to locate the hidden platform in the experiment, which suggests that the search strategy used by the MPDG rats might be considered more efficient than that used by the MG rats. However, another study showed that testing in a water maze task revealed no significant effects of any dose including 10 mg/kg of methylphenidate on learning (McFayden et al., 2002). The aforementioned different results may be due to the differences in animal species, medicine administration, etc.

In this study, methylphenidate might improve hippocampal functions including learning and memory. Sometimes the rats were far from accurate target location, but they had less blind movement more quickly, their looking for the hidden platform ability was significantly improved. Thus, we conclude that methyl-phenidate might play a positive role in improving spatial learning ability of the SH rats by adding its straight search strategy use in MWM test.

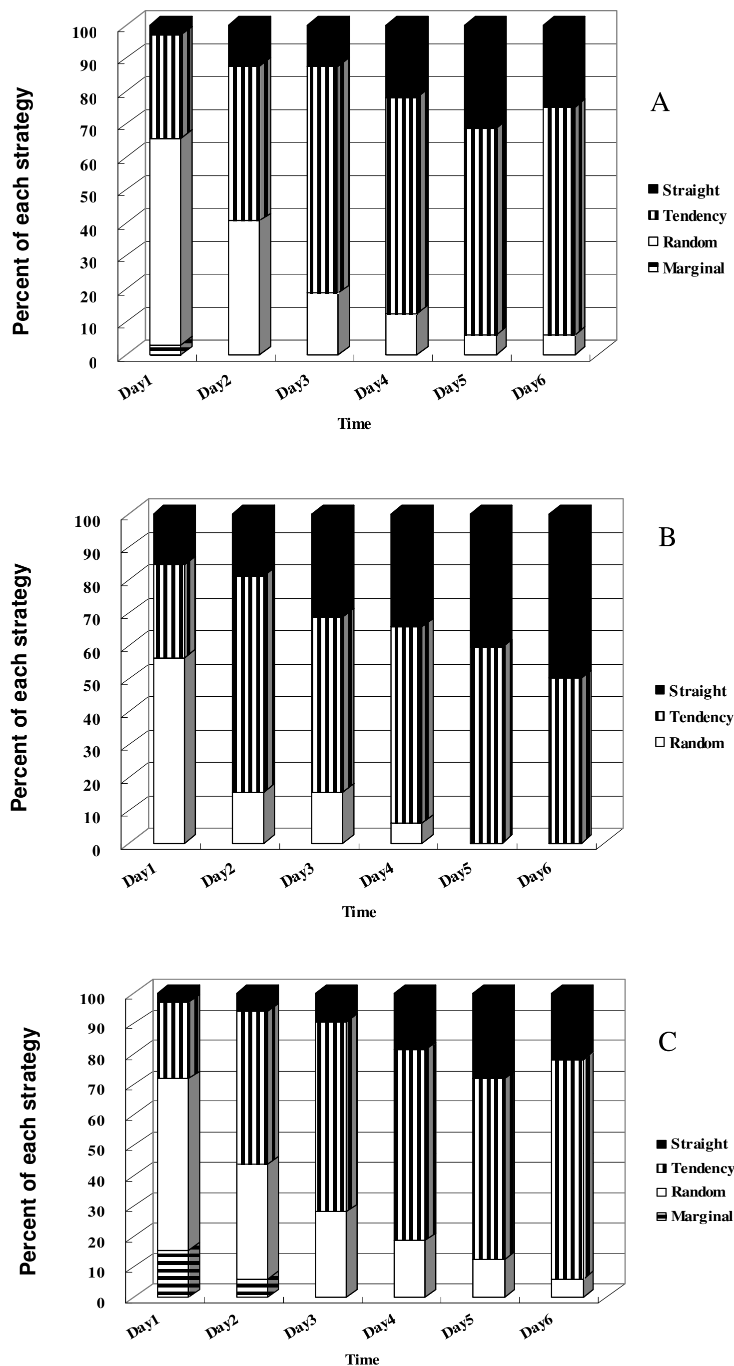

Figure 2. Percentage of each search strategy used by each group over 6 days of trials. Representative traces were shown for straight strategy (black), tendency strategy (vertical stripe), random strategy (white), and marginal strategy (horizontal stripe) in the (A) Model group, (B) Methylphenidate group, and (C) Control group, respectively. During the training in the Morris water maze, all the rats were more and more in favour of using the straight strategy and the tendency strategy as well to search the hidden platform. The MG used the straight strategy with a higher percent compared to the CG ($P<0.01$), meanwhile, the MPDG used the same strategy with a higher percent as compared to the MG ($P<0.001$) during the whole training. Specifically, the MPDG rats used the straight strategy more frequently than the MG rats did on day 1 ($P<0.01$), day 3 ($P<0.01$) and day 6 ($P<0.001$), respectively, however, daily comparisons were not significant between the MG and the CG.

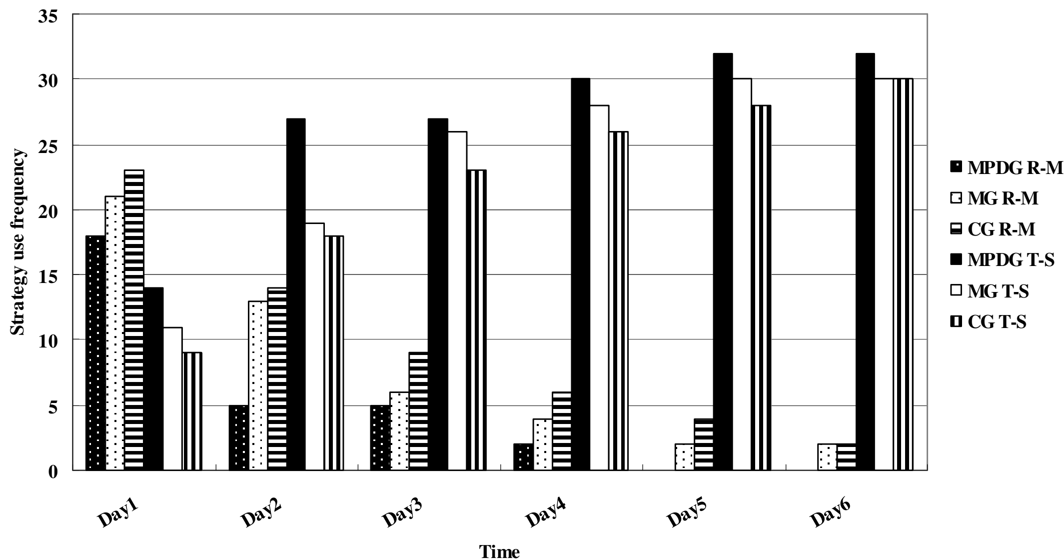

Figure 3. Frequency of the random-marginal strategy and the tendency-straight strategy used by each group over 6 days of trials.
Traces were shown for the R-M strategy (black with white dots) and the T-S strategy (black) in the MPDG; the R-M strategy (white with black dots) and the T-S strategy (white) in the MG; the R-M strategy (horizontal stripe) and the T-S strategy (vertical stripe) in the CG, respectively. From the 2nd day of training, the frequency of the T-S strategy was significantly increased in the three groups, respectively ($P<0.05$). R-M: Random-marginal; T-S: tendency-straight; MPDG: methylphenidate hydrochloride group; MG: model group; CG: control group.

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ABBREVIATIONS

ADHD, Attention-deficit/hyperactivity disorder; **SH,** spontaneously hypertensive; **WKY,** Wistar Kyoto; **MWM,** Morris water maze; **MG,** model group; **MPDG,** methylphenidate hydrochloride group; **CG,** control group; **T-S,** tendency-straight; **R-M,** random-marginal.

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Full Length Research Paper

Effects of olanzapine versus risperidone on body mass index, serum leptin and lipid profile in schizophrenic patients

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Weight gain is a common adverse effects associated with the use of some atypical antipsychotic drugs contributing to morbidity and poor adherence to treatment. Also, the antipsychotic treatment associated with increased levels of the hormone leptin and dyslipidemia. This study was conducted to investigate the influence of the two atypical antipsychotic drugs (olanzapine and risperidone) on body mass index (BMI), leptin and lipid profile in schizophrenic patients. Of the 88 patients enrolled, only 65 patients completed this study. Thirty four patients on olanzapine at a mean daily dose of 9.85±3.74 mg and 31 patients on risperidone at a mean daily dose of 6.58±2.07 mg. Fourty apparently healthy volunteers with age, sex and BMI matched to the patients were collected as a control group. BMI and serum levels of leptin and lipid profile were estimated before treatment and at 4 and 8 weeks of treatment and compared with the results of healthy volunteers for the same parameters. At baseline, there were no significant differences between olanzapine, risperidone and control groups in terms of BMI, leptin, cholesterol, high density lipoprotein (HDL), triglycerides, low density lipoprotein (LDL) and atherogenic index. After treatment with olanzapine and risperidone, the two drugs causes significant increase in the levels of BMI, leptin, cholesterol, triglycerides, LDL and atherogenic index after 4 and 8 weeks of treatment while HDL was reduced by treatment with both drugs. This study demonstrated that olanzapine and risperidone treatment for 8 weeks caused significant increase in BMI, serum leptin levels and also causes deleterious effects on lipid profile. There were also no significant differences between olanzapine and risperidone with regard to their effects on leptin, cholesterol, HDL, LDL and atherogenic index.

Key words: Schizophrenia, olanzapine, risperidone, leptin, body mass index (BMI), lipid profile.

INTRODUCTION

Schizophrenia is a psychosis characterized by delusions, hallucinations and lack of insight, with disturbed behavior and disordered thinking (Boon et al., 2006). Antipsychotics have become the first-line treatment for schizophrenia as well as for other psychotic disorders (De-Oliveira and Juruena, 2006).

The second generation antipsychotic (SGA) drugs such as olanzapine, risperidone, quetiapine and ziprazidone are effective in treating both the positive and negative symptoms of schizophrenia (Kelleher et al., 2002). These

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advantages have led to an increasing use of SGAs as the first line therapy for schizophrenia. However, SGAs have been linked to several forms of morbidity, including obesity, hyperlipidemia and type 2 diabetes mellitus (Jin et al., 2004; Melkersson and Dahl, 2004; Bergman and Ader, 2005).

Medication-induced weight gain has been associated with a lower quality of life (Allison et al., 2003) and non-compliance (Weiden et al., 2004), which increases the risk for relapse (Robinson et al., 1999). The mechanism of weight-gain associated with antipsychotic drugs is not well understood and is also probably multifac- torially determined (Brady, 1989). Several possibilities are however suggested, for example, that the sedative effects of antipsychotics lead to less physical activity and therefore lowered calorie utilization and that thirst due to anticholinergic activity of antipsychotics increases the consumption of high-calorie beverages (Stanton, 1995). The serotonergic blockade of antipsychotics, through an antagonism of the serotonin-2C (5-HT_{2c}) receptors, has also been discussed as a possible cause of increased food intake and weight induced by antipsychotics (Tecott et al., 1995; Bonhaus et al., 1997).

Leptin, an adipocyte-derived hormone, is considered one of the main peripheral signals that affect food intake and body weight (Prolo et al., 1998). After leptin is released by the adipose tissue into the blood stream, it crosses the blood-brain barrier and binds to the hypothalamic leptin receptors in the arcuate nucleus, giving information about the status of the body energy stores (Sahu, 2003). The persistent elevation in leptin levels may reflect a loss of the normal inhibitory control of leptin on body mass in patients with schizophrenia (Zhang et al., 2004). Administration of leptin into the arcuate nucleus results in decreased food consumption while leptin deficiency leads to increased food intake (Haupt et al., 2005). Many studies indicate that serum leptin levels increased after administration of antipsychotics to patients with schizophrenia (Fitzgerald et al., 2003; Atmaca et al., 2003; Zhang et al., 2004; Maayan and Vakhrusheva, 2010).

The aim of the study was to investigate the effects of atypical antipsychotics olanzapine and risperidone on body mass index (BMI), leptin and lipid profile in patients with schizophrenia in comparison to controls.

METHODOLOGY

Schizophrenic patients

Of 88 patients enrolled, only 65 patients completed this study. Thirty four patients on olanzapine (24 males and 10 females) in a mean daily dose of 9.85±3.74 mg and 31 patients on risperidone (20 males and 11 females) in a mean daily dose of 6.58±2.07 mg. Twenty three patients dropped out from the study. Diagnosis of schizophrenia was made using Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-1994) criteria. Each patient underwent diagnostic evaluation by one specialist psychiatrist on the basis of a semistructural interview to determine DSM-IV diagnosis. All patients or their relatives gave written informed consent. Patients with the following criteria were excluded from the study (patients with drugs abuse, patients with other diseases especially diabetes and any other endocrinological, immunological disorders or patients treated with other antipsychotics, other exclusion criteria include pregnant and lactating women).

Forty apparently healthy volunteers (27 males and 13 females) without previous history of any psychiatric disorder were recruited as controls with approximately age, sex and BMI matching to the patients groups. The controls group was judged to be free of any illnesses by history and clinical examination.

Study protocol

Newly diagnosed or drug free for at least 4 weeks schizophrenic patients put according to their conditions on either olanzapine or risperidone (two groups) and fasting blood samples were initially taken from the two groups for measuring leptin, lipid profile and BMI were calculated according to special equation. Then the patients continue on this therapy for 8 weeks. By the end of 4 and 8th weeks, another blood sample was taken from both groups and assessment of the same parameters (leptin, lipid profile and BMI) were done. From the control group, a fasting blood sample was taken and assay of the same parameters were done.

Sampling

Five milliliters venous blood was withdrawn from schizophrenic patients using disposable syringe at about 8.5 to 10 a.m. and after overnight fasting. The blood was allowed to clot in a plain tube at room temperature, and then the serum was separated by centrifugation at 3000 rpm for 10 min and kept frozen bending analysis. Samples from the control subjects were collected and processed in the same way.

Analytical methods

1) The MBI was calculated by dividing the weight (kg) by the squared height (m) (Saddichha et al., 2007).

$BMI = Weight\ (kg)/Height^2\ (m)$.

2) Serum leptin concentration were analyzed using immunoenzymatic assay for the *in vitro* quantitative measurement of human leptin in serum and plasma (direct sandwich ELISA kit, GenWay Biotech, Inc.USA).
3) Serum cholesterol, high density lipoprotein (HDL) and triglycerides were determined by enzymatic procedures using BIOLAB kit (France), whereas low density lipoprotein (LDL) was measured using Friedewald equation. Atherogenic index (AI) was calculated by the following equation:

$AI = TC/HDL$

Statistical methods

Data are presented as mean±standard deviation (SD), and were analyzed using analysis of variance (ANOVA), t-test and Duncan test to compare the results of the three groups. P-value of less than 0.05 is considered as significantly different.

RESULTS

The characteristics of all schizophrenic patients and control are shown in Table 1. The difference in mean±SD of baseline variables between olanzapine, risperidone and control group are shown in Table 2. The difference in mean±SD of variables before and after 4 weeks treatment with olanzapine are shown in Table 3. The difference in mean±SD of variables before and after 4 weeks treatment with risperidone are shown in Table 4. The difference in mean±SD of variables before and after 8 weeks treatment with olanzapine are shown in Table 5. The difference in mean±SD of variables before and after 8 weeks treatment with risperidone are shown in Table 6. Comparison of the net difference in mean±SD of variables before and after 4 weeks treatment between olanzepine and resperidone group (After 4 weeks baseline) are shown in Table 7. Comparison of the net difference in mean±SD of variables before and after 8 weeks treatment between olanzepine and resperidone group (After 8 weeks – baseline) are shown in Table 8.

DISCUSSION

This study investigated the effects of two atypical antipsychotics (olanzapine and risperidone) on BMI, the serum levels of leptin and lipid profiles.

This study indicated that at baseline, there were no significant differences among olanzapine, risperidone and control groups in terms of BMI, leptin, cholesterol, HDL, triglycerides, LDL and AI as shown in Table 2. Both olanzapine and risperidone cause a significant increase in BMI after 4 and further after 8 weeks of treatment with these drugs as shown in Tables 3, 4, 5 and 6. Previous studies with these drugs reached similar results and reported that BMI increased significantly after 4 weeks (Gothelf et al., 2002) and 8 weeks of treatment (Garyfallos et al., 2003). Of the two drugs, olanzapine causes a more significant increase in BMI than risperidone as shown in Tables 7 and 8 and these finding were in agreement with the results of other studies (Garyfallos et al., 2003; Saddichha et al., 2007).

Regarding leptin, previous studies have reported an increase in circulating leptin in patients treated with olanzapine (Eder et al., 2001; Atmaca et al., 2003; Ebenbichler et al., 2005) and risperidone (Fitzgerald et al., 2003; Zhang et al., 2004; Maayan and Vakhrusheva, 2010). The present study demonstrated that serum leptin was increased significantly after 4 weeks of therapy with both drugs in comparison to baseline values as shown in Tables 3 and 4. These results were in agreement with the results of Kraus et al. (1999), as they studied the effects of antipsychotic drugs (clozapine and olanzapine) on body weight and leptin over a period of 4 weeks, they reported that clozapine and olanzapine cause significant increase in serum leptin levels at week-4 of treatment. Hosojima et al. (2006) also in agreement with our study, reported that serum leptin in thirteen schizophrenic patients treated with olanzapine had increased significantly at week 4 of treatment in comparison to baseline levels.

This study also showed that at week-8 of treatment with both drugs, the serum leptin level decreased to levels less than that observed at week 4, but greater than those observed at baseline as shown in Tables 5 and 6. Our observation with regard serum leptin level over 8 weeks of treatment goes with the finding of other studies.

A prospective 10-week clozapine trial conducted by Brömel et al. (1998) reported that leptin levels peaked early in treatment at week-2 followed by subsequent decrease, and the study of Monteleone et al. (2002) who prospectively measured plasma leptin levels in 22 chronic resistant schizophrenic patients treated with clozapine monotherapy, blood samples were taken at baseline and after 1, 2, 4, 6, 8, 12, 16, 24 and 32 weeks. These authors found that plasma leptin concentrations doubled after only 2 weeks of clozapine administration. By the end of week 4, leptin levels abruptly fell to levels more strictly related to body weight changes. Although statistically not significant, olanzapine causes greater increase in serum leptin levels than risperidone as shown in Tables 7 and 8 and this result was in agreement with the result of other investigators (Atmaca et al., 2002).

The effects of olanzapine and risperidone on lipid profile is prominent and both drugs cause significant increase in cholesterol, triglycerides, LDL and AI (TC/HDL) after 4 and 8 weeks treatment as shown in Tables 3, 4, 5 and 6 and the level of HDL were decreased with both drugs in the same treatment periods, but the reduction in HDL was not significant except the effect of olanzapine at week 8 of treatment. These results were in agreement with other studies which investigated the effects of olanzapine and risperidone on lipid profile. In a study conducted by Tschoner et al. (2009) on 28 patients with schizophrenic disorders treated with 6 antipsychotic disorders for 4 weeks, they found that olanzapine caused a significant increase in cholesterol, triglycerides, LDL, while HDL decreased by the end of treatment period. In other study conducted on 29 schizophrenic patients on clozapine or risperidone monotherapy for 6 weeks. Idonije et al. (2012) concluded that by the end of 6 weeks treatment, there was a significant increase in serum total cholesterol, triglycerides and very-low-density lipoprotein (VLDL) and a significant decrease in HDL in both treatment groups as compared to the control. Ozguven et al. (2001) studied the metabolic effects of olanzapine and quetiapine on 30 patients over 6 weeks, and they reported that olanzapine causes a significant increase in cholesterol and LDL by the end of the 6-week treatment.

Of the two drugs, olanzapine causes more increase in serum levels of cholesterol, triglycerides, LDL and AI and

Table 1. Patients and control characteristics and drug treatment.

Variable	Mean±SD			p-value
	Olanzapine (n=34)	Risperidone (n=31)	Control (n=40)	
Number	34	31	40	-
Age (year)	31.51±7.86	32.37±7.77	30.9±6.42	0.7
Weight (kg)	66.8±7.08	69.91±8.86	68.22±5.82	0.2
Sex (male/female)	24/10	20/11	27/13	-

Table 2. Difference in mean±SD of baseline variables between olanzapine, risperidone and control group.

Variable	Mean±SD			p-value
	Olanzapine (n=34)	Risperidone (n=31)	Control (n=40)	
Leptin (ng/ml)	12.16±7.66	14.67±10.69	10.92±1.52	0.1
BMI (kg/m^2)	23.94±2.77	24.28±2.50	24.31±2.81	0.8
Cholesterol(mmol/l)	5.03±0.82	4.75±0.89	4.85±0.81	0.3
HDL (mmol/l)	1.43±0.34	1.35±0.29	1.28±0.17	0.07
Triglyceride (mmol/l)	1.38±0.77	1.51±0.87	1.47±0.40	0.7
LDL (mmol/l)	2.94±0.76	2.76±0.83	2.98±0.57	0.4
Atherogenic index (AI)	3.51±0.86	3.60±1.14	3.87±0.91	0.7

Table 3. Difference in mean±SD of variables before and after 4 weeks treatment with olanzapine.

Variable	Mean±SD		p-value
	Before treatment with olanzapine (n=34)	After 4 weeks treatment with olanzapine (n=34)	
Leptin (ng/ml)	12.16±7.66	16.94±10.15	<0.0001
BMI (kg/m^2)	23.94±2.77	25.14±2.54	<0.0001
Cholesterol(mmol/l)	5.03±0.82	5.53±1.10	<0.001
HDL (mmol/l)	1.43±0.34	1.34±0.32	0.06
Triglyceride (mmol/l)	1.38±0.77	1.65±0.71	0.001
LDL (mmol/l)	2.94±0.76	3.38±1.00	0.001
Atherogenic index (AI)	3.51±0.86	4.19±1.08	<0.0001

Table 4. Difference in mean±SD of variables before and after 4 weeks treatment with risperidone.

Variable	Mean±SD		p-value
	Before treatment with risperidone (n=31)	After 4 weeks treatment with risperidone (n=31)	
Leptin (ng/ml)	14.67±10.69	17.49±11.07	0.002
BMI (kg/m^2)	24.28±2.50	24.95±2.60	<0.0001
Cholesterol (mmol/l)	4.75±0.89	5.18±0.93	0.005
HDL (mmol/l)	1.35±0.29	1.32±0.23	0.4
Triglyceride (mmol/l)	1.51±0.87	1.77±1.10	0.01
LDL (mmol/l)	2.76±0.83	3.09±0.96	<0.0001
Atherogenic index (AI)	3.60±1.14	3.99±1.03	0.007

Table 5. Difference in mean±SD of variables before and after 8 weeks treatment with olanzapine.

Variable	Mean±SD		*p*-value
	Before treatment with olanzapine (n=34)	After 8 weeks treatment with olanzapine (n=34)	
Leptin (ng/ml)	12.16±7.66	15.38±10.72	0.002
BMI (kg/m^2)	23.94±2.77	25.85±2.36	<0.0001
Cholesterol(mmol/l)	5.03±0.82	6.11±1.20	<0.0001
HDL (mmol/l)	1.43±0.34	1.25±0.33	0.005
Triglyceride (mmol/l)	1.38±0.77	2.05±1.24	<0.0001
LDL (mmol/l)	2.94±0.76	3.85±1.14	<0.0001
Atherogenic index (AI)	3.51±0.86	4.77±1.13	<0.0001

Table 6. Difference in mean±SD of variables before and after 8 weeks treatment with risperidone.

Variable	Mean±SD		p-value
	Before treatment with risperidone (n=31)	After 8 weeks treatment with risperidone (n=31)	
Leptin (ng/ml)	14.67±10.69	15.24±11.33	0.5
BMI (kg/m^2)	24.28±2.50	25.49±2.63	<0.0001
Cholesterol(mmol/l)	4.75±0.89	5.62±0.95	<0.0001
HDL (mmol/l)	1.35±0.29	1.30±0.18	0.2
Triglyceride (mmol/l)	1.51±0.87	1.83±0.80	0.006
LDL (mmol/l)	2.76±0.83	3.51±1.04	<0.0001
Atherogenic index (AI)	3.60±1.14	4.38±1.06	<0.0001

Table 7. The net difference in mean±SD of variables before and after 4 weeks treatment between olanzepine and risperidone group.

Variable	Mean±SD		p-value
	Olanzapine (n=34)	Risperidone (n=31)	
Leptin (ng/ml)	4.78±5.06	2.82±4.59	0.1
BMI (kg/m^2)	1.20±0.76	0.67±0.72	0.006
Cholesterol(mmol/l)	0.49±0.77	0.43±0.79	0.7
HDL (mmol/l)	-0.08±0.25	-0.03±0.25	0.4
Triglyceride (mmol/l)	0.27±0.41	0.26±0.57	0.9
LDL (mmol/l)	0.44±0.74	0.32±0.43	0.4
Atherogenic index (AI)	0.60±0.65	0.39±0.74	0.2

more decrease in HDL than risperidone, although the differences were not significant (except the effect on triglyceride at week 8 of treatment was significant) as shown in Tables 7 and 8. These results were in agreement with the results of the study conducted by Lee et al. (2010) who investigated the metabolic profile of first and second generation antipsychotics on 99 schizophrenic patients. They found out that patients on olanzapine had the greatest increase in cholesterol and triglycerides among all antipsychotics (including risperidone).

Conclusions

This study demonstrated that olanzapine and risperidone treatment for 8 weeks caused significant increase in BMI, serum leptin levels and also causes deleterious effects on lipid profile. There were also no significant differences between olanzapine and risperidone with regard to their

Table 8. The net difference in mean±SD of variables before and after 4 weeks treatment between olanzepine and risperidone group.

Variable	Mean±SD		p-value
	Olanzapine (n=34)	Risperidone (n=31)	
Leptin (ng/ml)	3.22±5.53	0.57±5.16	0.05
BMI (kg/m^2)	1.91±1.08	1.21±0.95	0.008
Cholesterol(mmol/l)	1.07±1.04	0.87±0.89	0.3
HDL (mmol/l)	-0.17±0.33	-0.05±0.25	0.1
Triglyceride (mmol/l)	0.67±0.66	0.31±0.59	0.02
LDL (mmol/l)	0.91±1.06	0.74±0.73	0.4
Atherogenic index (AI)	1.19±0.96	0.78±0.97	0.09

effects on leptin, cholesterol, HDL, LDL and AI.

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UPCOMING CONFERENCES

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