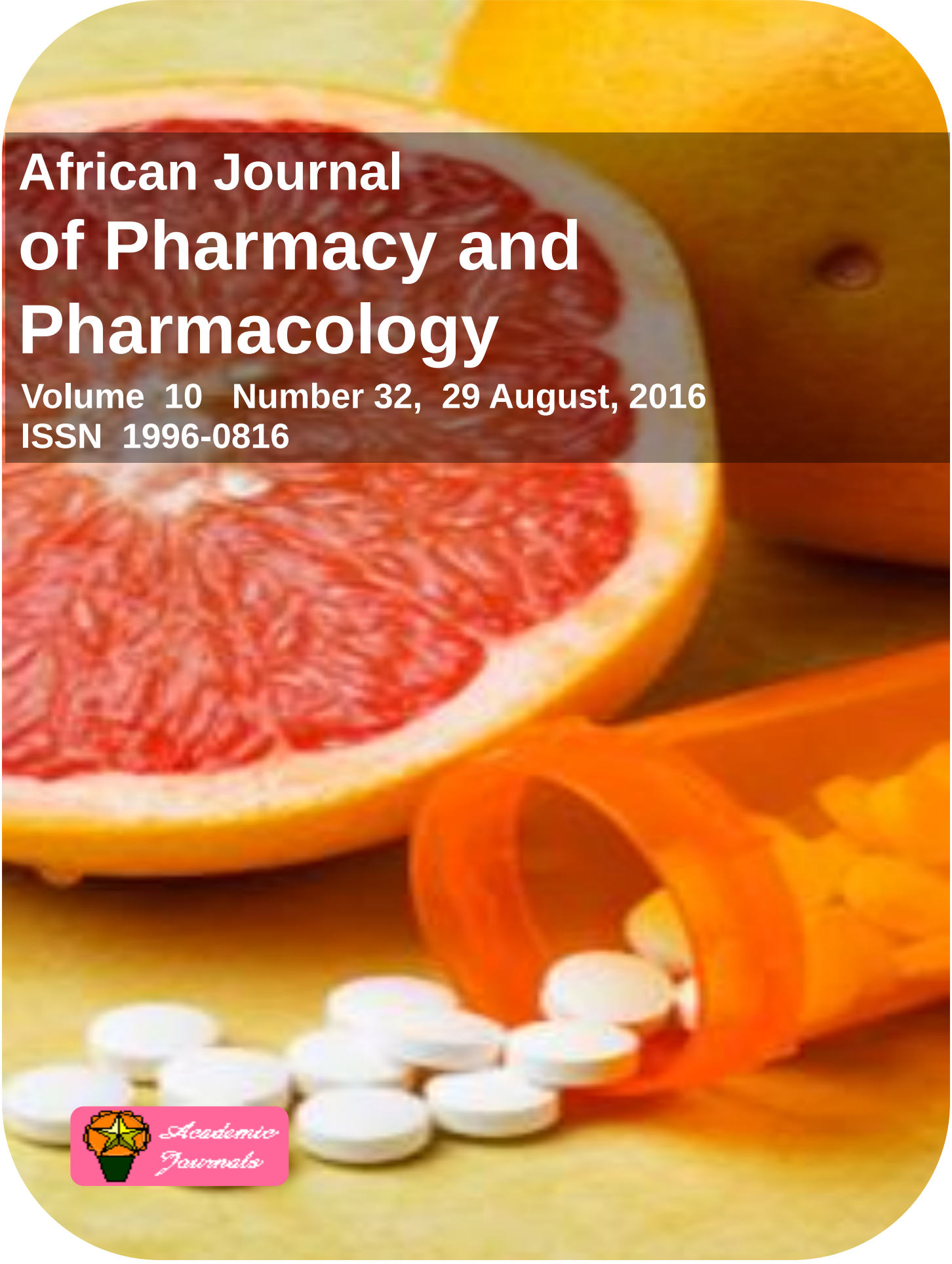

African Journal of Pharmacy and Pharmacology

Volume 10 Number 32, 29 August, 2016
ISSN 1996-0816

Academic Journals

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African Journal of Pharmacy and Pharmacology

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academicJournals

Vol. 10(32), pp. 657-664, 29 August, 2016
DOI: 10.5897/AJPP2015.4434
Article Number: 5FBE11D60401
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Study of antidiarrheal and anthelmintic activity methanol extract of *Commelina benghalensis* leaves

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Received 21 August 2015; Accepted 14 October, 2015

The objective of this study was to examine the restorative effects of methanol extract of *Commelina benghalensis* leaves in antidiarrheal activity in Swiss albino mice model and anthelmintic activity in *Tubifex tubifex*. Antidiarrheal activity study in Swiss albino mice were divided into four groups. Group I was treated as the control group and received 10 ml/kg of 2% Tween-80 orally; Group II served as a positive control and took standard drug (loperamide) in 5 mg/kg orally; Groups III and IV were the test groups which received the methanol extract of *C. benghalensis* orally at 200 and 400 mg/kg, respectively. Dependent upon the model comprehensive weight of dry feces, aggregate weight of wet defecation, length of intestinal travel and intestinal weight were gathered. At long last, information were investigated using restricted analysis of variance (ANOVA) took after by Dunnett test. Anthelmintic action on aquarium worm *T. tubifex* by utilizing three focuses of 10, 5, and 2.5 mg/ml of *C. benghalensis* movement was assessed by *in vitro* system. The property of the extract on defecation, intestinal transit and intestinal fluid accumulation (antienteropooling) were assessed in castor oil induced diarrhea. Leaves extract of *C. benghalensis* at 200 and 400 mg/kg exhibited significant ($p<0.05$, 0.01 and 0.001) and dose dependent antidiarrheal probable in castor oil induced diarrhea. The diarrhea was inhibited by 27.59 and 55.17 at doses of 200 and 400 mg/kg in that order. The extract were found to have an antienteropooling in castor oil affected test Swiss albino mice's by decreasing both weight and volume of intestinal substance, and obviously reducing the intestinal travel similar to that of the standard medication, loperamide (5 mg/kg). Anthelmintic activity of methanol extract *C. benghalensis* show the time taken for paralysis (12.38, 16.25 and 22.17 min) and time taken for death (21.39, 45.64 and 57.25 min) at doses of 10, 5, and 2.5, respectively. The methanol extract of *C. benghalensis* demonstrated antidiarrheal effect in Swiss albino mice model and anthelmintic action on aquarium worm *T. tubifex*. In addition, the extract was discovered to be safe at measurements of 1000, 2000, 3000, and 4000 mg/kg in mice model. The discoveries propose the legitimacy of the much lauded impact of *C. benghalensis* as antidiarrheal and anthelmintic specialists in expected home grown medication.

Key words: *Commelina benghalensis*, anti-diarrhea, Swiss albino mice, anthelmintic, *Tubifex tubifex*.

INTRODUCTION

Nature has been a recurrent source of pharmacologically dynamic atoms and curative herbs have been utilized by incalculable human eras (Zheng et al., 2015). *Commelina benghalensis* Linn. (Commelinaceae) is a perennial herb native to tropical Asia and Africa, commonly known as Bengal day flower or Dew flower. It is a large, straggling

annual herb up to 40 cm long with rooting at basal nodes and characterized by attractive small bluish-violet flowers. Leaves are ovate-elliptic or oblong, shortly triangular, bright green in color and 4 to 7 cm long. The spathes are green, funnel-shaped, compressed and about 1.5 cm long. Capsules are broadly ovoid-oblong and 4 to 5 mm long (Zheng et al., 2015).

C. benghalensis is utilized as a part of customary medication outline to treat different diseases. It is used for the treatment of headache, constipation, leprosy, fever, snake bite and jaundice (Hasan et al., 2008; Yusuf et al., 1994; Kirtikar and Basu, 1980). It is also used in the treatment of mouth thrush (Ssenyonga and Brehony, 1993), insanity (Tabuti et al., 2003), epilepsy (Okello and Ssegawa, 2007) and psychosis (Adjanohoun, 1993). In Lesotho, it is applied to treat infertility in women and in India it is used as bitter, laxative, anti-inflammatory, demulcent, emollient and depressant (Jayvir et al., 2007). In China, it is used as diuretic and febrifuge (Hong and Defllips, 2000). In Pakistan it is used as vegetable (Qaiser and Jafri, 1975). In Nepal, the paste of the plant is utilized to treat smolders and juice of the roots is utilized to treat acid reflux (Manandhar and Sanjay, 2000).

In a past study, antimicrobial action of watery extract of *C. benghalensis* was assessed (Sharma and Sharma, 2010). Potential calming and anxiolytic exercises of diverse portions of the plant are accounted for in the writing (Hasan et al., 2009). The plant is also reported with remarkable antioxidant, antitumor and anticancer, and thrombolytic activity (Zheng et al., 2015; Mbazima et al., 2008; Rahman et al., 1999; Chowdhury et al., 2015). Protective activity of the roots extract against paracetamol induced hepatic damage in Wistar rats has been reported (Paresh and Chanda, 2008). Phytochemical investigations on *C. benghalensis* have revealed the existence of alkaloid, volatile oil, wax (Raju et al., 2007), vitamin C, vitamin A and *β*-carotene (Guerrant et al., 2001). Diarrhea can be characterized as a modification in the ordinary solid discharge, portrayed by a circumstance in which a grown-up every day stools surpasses 300 g and contains 60 to 95% water (Zavala et al., 1998). Looseness of the bowels is one of the primary drivers of newborn child passing particularly in underdeveloped nation (Galheigo et al., 2015). It represents more than 5 million passing in overall every year in newborn children and offspring of under 5 years. Therapeutic plants are potential wellsprings of antidiarrheal medications (Van et al., 2015; Chowdhury et al., 2015).

In Bangladesh, one third of the total child death burden is due to diarrhea (Patzi et al., 2015). Use of traditional medicines to combat the episodes of diarrhea has been emphasized by WHO in its Diarrhea Control Program (Wansi et al., 2007). Medicinal plants have been reported for their efficiency in the treatment of diarrhea, thanks to the contribution of many researchers (Dooley et al., 2015).Gastrointestinal nematodes (GIN) cause genuine monetary misfortunes and is the most imperative element restricting sheep generation around the world (Prada et al., 2014; Valcarcel et al., 2015; Debebe et al., 2015). Anthelmintic are medications that demonstrate or systemically to kill grown-up helminths or formative stages that attack organs and tissues (Lou et al., 2014). *T. tubifex* is a provincially to oust worms from the gastro-intestinal tract cosmopolitan naidid annelid sensu (Lou et al., 2014) representing one of the major components of the benthic fauna in freshwater communities (Schmelz et al., 2015). Also present in polluted waters, *T. tubifex* is widely used in laboratories for ecotoxicology research (Mendez et al., 2014) and as a model organism for the study of annelid development (Mendez et al., 2013). *T. tubifex* is characterized by considerable variability in its morphological features (Urbisz et al., 2015) and by a mixed reproductive strategy, with parthenogenesis (Marotta et al., 2014), self-fertilization (Gavrilov, 1935), and biparental reproduction through cross-mating (Sarker et al., 2015).

The aim of the present study is to identify the antidiarrheal activity by using different experimental methods and anthelmintic activity of methanol extract of *C. benghalensis* leaves using *T. tubifex* worm. However, no earlier studies have been conducted experimentally to characterize the antidiarrheal and anthelmintic effect of this leaves.

MATERIALS AND METHODS

Plant collection and identification

The leaves of *C. benghalensis* were collected from Chittagong, Bangladesh in the month of October, 2014. The leaves were taxonomically identified by Dr. Shaikh Bokhtear Uddin (Associate Professor, Department of Botany, University of Chittagong, Bangladesh). A voucher specimen (Accession No. Pharm P&D 71/09-15/30) was deposited at the Department of Pharmacy, International Islamic University Chittagong, Bangladesh for further reference.

Extract preparation

The leaves were dried for a period of 2 weeks under shade and ground. The ground leaves (250 g) were soaked in sufficient amount of methanol for one week at room temperature with occasional shaking and stirring, and then filtered through a cotton plug followed by Whitman filter paper number 1. The solvent was evaporated under reduced pressure at room temperature to yield semisolid. The extract was then preserved in a refrigerator till

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further use.

Experimental animals

Swiss albino mice, weighing about 25 to 30 g, were collected from Jahangir Nagar University, Savar, Bangladesh. The animals were provided with standard laboratory food and distilled water and maintained at natural day-night cycle having proper ventilation in the room. All the experiments were conducted in an isolated and noiseless condition. The study protocol was approved by the P&D Committee, Department of Pharmacy, International Islamic University of Chittagong, Bangladesh (Grant No. Pharm P&D 71/09-15/30). The animals were acclimatized to laboratory condition for 7 days prior to experimentation.

Experimental worms

Experimental worms were collected from local aquarium shop. Then, authenticated by local zoologist (Authentication No. Wb2015).

Chemicals and reagents

Loperamide (Square Pharmaceuticals Ltd., Bangladesh), castor oil (WELL's Heath Care, Spain), normal saline solution (0.9% NaCl) and charcoal meal (10% activated charcoal in 5% gum acacia), and albendazole were used for anthelmintic activity tests. All other reagents were of analytical grade.

Preparation of test doses

The extracts were suspended in the vehicle. Various strengths were prepared from a stock solution of 40 mg/ml. The solutions were prepared, and the freshly prepared solutions were administered orally.

Acute toxicity study

For acute toxicity study, forty Swiss albino female mice were used. According to the method of Walum, mice were divided into four groups of five animals each (Walum, 1998). Different doses (1000, 2000, 3000 and 4000 mg/kg) of methanol extract of *C. benghalensis* leaves were administered by stomach tube. Then, the animals were observed for general toxicity signs.

***In vivo* antidiarrheal activity**

Castor oil-induced diarrhea

The experiment employed the method described by Awouters et al. (1978). Mice were fasted for 18 h before the test with free access to water and divided into four groups of five animals each. Group I treated as control (saline 2 ml/kg body weight intraperitoneally), Group II received standard drug (loperamide 5 mg/kg body weight p.o.), Groups III and IV received methanol extract of *C. benghalensis* (200 and 400 mg/kg body weight p.o.). Then, 1 h later, castor oil was administered orally to these animals to induce diarrhea. The mice's were then housed singly in cages lined with white blotting paper. The papers were changed every hour. The total number of both dry and wet feces excreted were counted every hour for a period of 4 h and compared with the control group. The total number of diarrheal feces of the control group was considered 100%.

Castor oil induced enteropooling

Intraluminal fluid accumulation was determined by the method of Robert et al. (1976). Mice were fasted for 18 h and divided into five groups of four animals each. Group I served as control (saline 2 ml/kg body weight intraperitoneally), Group II received standard drug (loperamide 5 mg/kg body weight ip), Groups III and IV received methanol extract of *C. benghalensis* (200 and 400 mg/kg body weight po). Then, 1 h later, castor oil was administered orally to these animals to induce diarrhea. Two hours later, the mice were sacrificed by overdose of chloroform anesthesia, and the small intestine was ligated both at the pyloric sphincter and at the ileocecal junctions and dissected out. The small intestine was weighed. The intestinal contents were collected by milking into a graduated tube and the volume was measured. The intestines were reweighed and the differences between full and empty intestines were calculated.

Gastrointestinal motility test

This experiment was carried out by the method described by Mascolo et al. (1994). Mice were fasted for 18 h and divided into four groups of five animals each. Castor oil was administered orally to these animals to induce diarrhea. One hour later, Group I received saline 2 ml/kg body weight intraperitoneally, Group II received standard drug (loperamide 5 mg/kg body weight ip), Groups III and IV received methanol extract of *C. benghalensis* (200 and 400 mg/kg body weight po). One hour after intraperitoneal administration of treatments, animals received 1 ml of charcoal meal (10% charcoal suspension in 5% gum acacia) orally. One hour later, the animals were sacrificed by overdose of chloroform anesthesia and the distance traveled by the charcoal meal from pylorus to caecum was measured and expressed as a percentage of the total distance of the intestine.

***In-vitro* anthelmintic assay**

The anthelmintic activity of methanol extract of *C. benghalensis* was carried out as per the procedure of Ajaiyeoba et al. (2001) with some minor modifications. The aquarium worm *T. tubifex* were used in the present study, because it has anatomical similarity and belongs to the same group of intestinal worm, that is, annelid (Verma et al., 2013; Raju et al., 2013; Rajagopal et al., 2013). The worms were collected from the local market of Chittagong, and average size of the worms, 2 to 2.5 cm in length were used for the study. The standard drug levamisole (1 mg/ml) and three different concentrations of methanol extract of *C. benghalensis* (2.5, 5 and 10 mg/ml) in double distilled water (Satish and Ravindra, 2009; Iqbal et al., 2001) were prepared freshly and used for the study of anthelmintic activity. One group composed of water and it was considered as the controlled group. The anthelmintic activity was determined at two different stages 'time of paralysis' and 'time of death' of the worms. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Death was concluded when the worms lost their motility followed with fading away of their body colors (Grime et al., 2006). Death was also confirmed by dipping the worms in slightly warm water. The mortality of parasite was assumed to have occurred when all signs of movement had ceased (Temjenmongla and Yadav, 2005).

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one way factorial ANOVA tests

Table 1. Effect of methanol extract of leaves of C. *benghalensis* on castor oil (0.5 ml) induced diarrhea in Swiss albino mice.

Group	Treatment	Total number of feces	Inhibition of defecation (%)	Total number of diarrheal feces	Inhibition of diarrhea (%)
I	Saline (2 ml/kg p.o.)	13.4±0.245	-	5.8±0.2	-
II	Loperamide (5 mg/kg p.o.)	5.4±0.4[c]	59.70±2.99	2.2±0.374[b]	62.07±6.45
III	MECB (200 mg/ml p.o.)	8.8±0.2[c]	34.33±1.49	4.2±0.2[b]	27.59±3.45
IV	MECB (400 mg/ml p.o.)	7±0.547[c]	47.76±4.09	3.2±0.2[c]	55.17±3.45

Values are mean ± SEM (n = 5); [a]p< 0.05, [b]p< 0.01 and [c]p < 0.001, Dunnett test as compared to negative control (saline). Statistical representation of the total number of feces and total number of diarrheal feces by *C. benghalensis* methanol extract, positive antidiarrheal control (Loperamide, 5 mg/kg p.o.) processed by paired t-test analysis (Dennett's test). Data were processed by paired t-test analysis by using SPSS for windows, version 16.0.

Table 2. Effect of methanolic extract of leaves of C. *benghalensis* on castor oil induced enteropooling in Swiss albino mice.

Group	Treatment	Volume of intestinal content (ml)	Weight of intestinal content (g)	Inhibition of intestinal content (%)
I	Saline (2 ml/kg p.o.)	0.24±0.013	0.628±0.033	-
II	Loperamide (5 mg/kg p.o.)	0.13±0.001	0.316±0.002[b]	48.2±0.40
III	MECB (200 mg/ml p.o.)	0.17±0.007	0.408±0.02[b]	33.11±3.64
IV	MECB (400 mg/ml p.o.)	0.15±0.005	0.334±0.01[b]	45.25±1.84

Values are mean ± SEM (n = 5); [a]p < 0.05, [b]p< 0.01 and [c]p < 0.001, Dunnett test as compared to negative control (saline). Statistical representation of the weight of intestinal content (g) by *C. benghalensis* methanol extract, positive antidiarrheal control (Loperamide, 5 mg/kg p.o.) processed by paired t-test analysis (Dennett's test). Data were processed by paired t-test analysis by using SPSS for windows, version 16.0.

using SPSS Data Editor for Windows, Version 16.0 (SPSS Inc., USA) followed by Dennett's tests on each group except the control group for anthelmintic. The results obtained were compared with the negative control group for antidiarrheal activity and P < 0.05, P < 0.01 and P < 0.001 was considered to be statistically significant in Dennett's tests. Statistical program GRAPHPAD PRISM® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) was used for graphical presentation.

RESULTS

Acute toxicity test

None of the animals showed behavioral, neurological or physical changes characterized by symptoms, such as reduced motor activity, restlessness, convulsions, coma, diarrhea and lacrimation at the limit dose of 4000 mg/kg for methanol extract of C. *benghalensis* during the observation period. In addition, no mortality was observed at the test dose. Thus, the median lethal dose (LD_{50}) of the plant extract was found to be greater than 4000 mg/kg.

In vivo antidiarrheal activity

Castor oil-induced diarrhea

In the castor oil-induced diarrhea conduct experiment, the leaves extract of *C. benghalensis* produced a noticeable antidiarrheal result in the mice's, as shown in Table 1. At doses of 200 and 400 mg/kg, the extract produced significant (p < 0.01) defecation. The total number of wet feces produced upon administration of castor oil decreased (4.2 ± 0.2, at 200 mg/kg and 3.2 ± 0.2, at 400 mg/kg) compared to the control group (5.8 ± 0.2) while loperamide decreased to 2.2 ± 0.374 at the dose of 5 mg/kg.

Castor oil induced enteropooling

Castor oil caused accumulation of water and electrolytes in intestinal loop. Treatment with the *C. benghalensis* extract (200 and 400 mg/kg) produced a significant and dose-dependent reduction in intestinal weight and volume (Table 2). The intestinal volume was decreased by 33.11±3.64 and 45.25±1.84% at doses 200 and 400 mg/kg, respectively. The standard drug, loperamide (5 mg/kg), also significantly inhibited (p < 0.01) intestinal fluid accumulation (48.2±0.40%).

Gastrointestinal motility test

The consequence of *C. benghalensis* extract on the intestinal transit is shown in Table 3. All doses of the extracts successful produced significant alteration in the percent of intestinal motility compared to the negative control. The negative control (saline) resulted in 84.85±2.88%

Table 3. Effect of methanolic extract of leaves of *C. benghalensis* on charcoal induced gut transit changes in Swiss albino mice.

Group	Treatment	Intestine cross by marker (%)	Inhibition (%)
I	Saline (2 ml/kg p.o.)	84.85±2.88	
II	Loperamide (5 mg/kg po)	45.35±1.72^{c}	43.6±2.14
III	MEHO (200 mg/ml po)	57.95±0.71^{b}	27.92±0.88
IV	MEHO (400 mg/ml po)	51.99±1.2^{c}	35.34±1.5

Values are mean ± SEM (n = 5); $^{a}p < 0.05$, $^{b}p< 0.01$ and $^{c}p < 0.001$, Dunnett test as compared to negative control (saline). Statistical representation of the % of intestine cross by marker by *C. benghalensis* methanol extract, positive antidiarrheal control (Loperamide, 5 mg/kg p.o.) processed by paired t-test analysis (Dennett's test). Data were processed by paired t-test analysis using SPSS for windows, version 16.0.

Table 4. Anthelmintic activity of methanol extract of *C. benghalensis*.

Treatment	Time taken for paralysis (min)	Time taken for Death (min)
Control (Water)	0	0.00
Levamisole (1 mg/ml)	3.3±0.38	6.5±0.76
C. benghalensis (10 mg/ml)	12.38±0.43^{b}	21.39±0.50^{b}
C. benghalensis (5 mg/ml)	16.25±0.48^{b}	45.64±1.01^{b}
C. benghalensis (2.5 mg/ml)	22.17±0.49^{b}	57.25±1.18^{b}

Values are mean ± SEM, (n = 3); $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$, Dennett's test as compared to positive control (Levamisole, 1 mg/ml). Statistical representation of the effective paralysis and dead time by *C. benghalensis* methanol extract, positive anthelmintic control (Levamisole, 1 mg/ml) processed by paired t-test analysis (Dennett's test). Data were processed by paired t-test analysis using SPSS for windows, version 16.0.

intestinal motility by the marker-charcoal meal. The 200 and 400 mg/kg oral dose of the extracts of *C. benghalensis* exhibited 57.95±0.7 and 51.99±1.2^{c} intestinal motility (Table 3). And the extracts significantly inhibited 27.92±0.88 and 35.34±1.5 % at all doses in intestinal motility. However, the standard drug, loperamide (5 mg/kg) demonstrated a significant inhibition (43.6±2.14%) in intestinal motility.

Anthelmintic activity

Consequences of the study were recorded as shown in Table 4 and Figure 1 as in the form of time required to get the following attacks of paralysis and at the end time required for complete death of parasite. From the aforementioned study, it was seen that the methanolic extract showed dose dependent anthelmintic activity as compared to a standard drug levamisole. Methanol extract *C. benghalensis* show the time taken for paralysis (12.38, 16.25, and 22.17 min) and time taken for death (21.39, 45.64, and 57.25 min) at dose 10, 5, and 2.5, respectively compared to a standard drug levamisole (1 mg/ml) that showed the time taken for paralysis (3.3±0.38 min) and time taken for death (6.5±0.76 min).

DISCUSSION

It is generally realized that castor oil is metabolized into ricinoleic corrosive in the gut which is in charge of looseness of the bowels generation (Darracq et al., 2015). The peristaltic action of small digestive tract is expanded if ricinoleate is introduced in the small digestive system, thus Na^{+} and Cl^{-} porousness changed in intestinal mucosa (Tariq et al., 2015). Emission of endogenous prostaglandin is empowered additionally by ricinoleate (Beubler and Juan, 1979; Sadraei et al., 2014). The leaves extract of *C. benghalensis* at 200 and 400 mg/kg measurements showed enormous decrease of the quantity of diarrheal and total feces which may be because of the initiation of prostaglandin biosynthesis with resultant abatement in emission of liquid into the lumen or may be because of advancement and ingestion of water and electrolytes in the gut. The standard drug, loperamide (5 mg/kg) also produced statistically significant ($p<0.01$) diarrheal inhibition (62.07%). The intestinal volume was decreased by 33.11 and 45.25% at doses of 200 and 400 mg/kg, respectively. The aforementioned hypothesis was further upheld by the inhibitory activity of the extract on intestinal charcoal supper motility. Methanol extract of *C. benghalensis* smothered the propulsive development or travel of charcoal supper through the gastrointestinal tract which fundamentally shows that the extract may have the probability to decrease the recurrence of stooling in diarrheal conditions.

Anthelmintic medications act quickly and specifically on neuromuscular transmission of nematodes. Three major

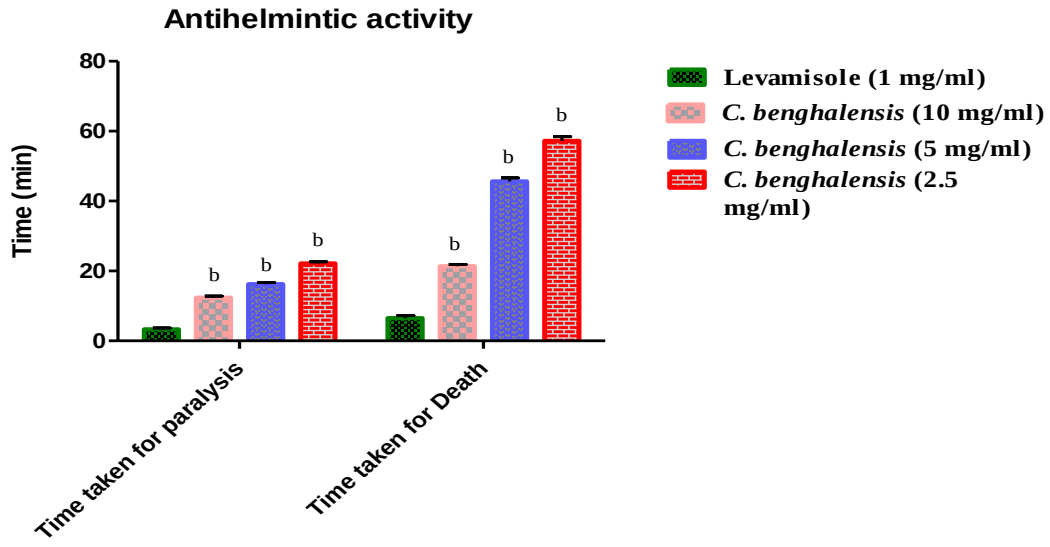

Figure 1. Anthelmintic activity of methanol extract of *C. benghalensis*. Values are mean ± SEM (n = 3); $^{a}p< 0.05$, $^{b}p < 0.01$ and $^{c}p< 0.001$, Dennett's test as compared to positive control (Levamisole, 1 mg/ml). Statistical representation of the effective paralysis and dead time by *C. benghalensis* methanol extract, positive anthelmintic control (Levamisole, 1 mg/ml) processed by paired t-test analysis (Dennett's test). Data were processed by paired t-test analysis using SPSS for windows, version 16.0.

groups of helminths (worms), the nematodes, trematodes, and cestodes- infect human (Mwale and Masika 2015; Pawluk et al., 2015; Romero et al., 2014). Nematodes are elongated roundworms that possess a complete digestive system, including both mouth and an anus. These cause the infections of the intestine as well as the blood and tissues. The trematodes (flukes) are leaf-shaped flatworms that are generally characterized by the tissues they infect. For example, they may be categorized as liver, lung, intestinal, or blood flukes. The cestodes or true tapeworms typically have a flat, segmented body and are attach to the hosts intestine. Levamisole are agonists at nicotinic acetylcholine receptors of nematode muscle and reason spastic loss of motion.

The methanol extract of *C. benghalensis* showed anthelmintic activity at a concentration of 10, 5 and 2.5 mg/ml. The anthelmintic effect of the extract is comparable with that of the effect produced by the standard drug levamisole (1 mg/ml) which also produced statistically significant ($p<0.01$) anthelmintic activity time taken for paralysis (3.3 min) and time taken for death (6.5 min). The activity was concentration dependent. The plant possesses significant anthelmintic activity at 10, 5, and 2.5 mg/ml concentration measured by time taken for paralysis (12.38, 16.25, and 22.17 min) and death of the earth worm (21.39, 45.64, and 57.25 min) in that order.

Conclusion

This study underpins that the methanol extract of *C. benghalensis* leaves are the planned sources of antidiarrheal and anthelmintic specialists in the conventional drug framework. In this case, studies are obliged to assist to distinguish the dynamic constituent(s) of the portions to comprehend the pharmacological activity of the antidiarrheal and anthelmintic impacts. The discoveries propose the legitimacy of the much lauded impact of *C. benghalensis* as antidiarrheal and anthelmintic specialists in expected home grown medication.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors express their thanks to the Jahangir Nagar University, Savar, Dhaka, Bangladesh, for providing experimental mice.

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academicJournals

Vol. 10(32), pp. 665-669, 29 August, 2016
DOI: 10.5897/AJPP2016.4634
Article Number: B94780160403
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Potential impact of urocortin I on sperm count, motility and sex hormone profiles in normal adult rats

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Received 22 July 2016: Accepted 19 August 2016

Urocortin I (Ucn I), Ucn II and Ucn III are corticotropin-releasing factor (CRF) - like peptides. Ucn I has a high affinity for CRF_1 and CRF_2 receptors. The function of Ucn in male reproduction has not yet been elucidated. Ucn I is present in rat testis with lower levels of Ucn II and III mRNA gene expressions. Therefore, it is suggested that Ucn I may play a role in the regulation of male reproductive action. This study is designed to find out the endocrine and reproductive effects of Ucn I in normal adult male rats through investigation of exogenous Ucn I effects on epididymal sperm count, sperm motility, testicular weight and sex hormone profiles. 24 normal adult male albino rats of 175 to 200 gm initial body weight were implemented in this study. Randomly, the rats were subdivided into four equal groups. Group (I): Vehicle-treated group in which control rats received 0.1 mL of normal saline for 42 days as Ucn I-treated groups. Rats in group II were divided into three equal subgroups. Group (IIA), (IIB) and (IIC): Ucn I-treated groups in which rats were given daily intraperitoneal injections of rat Ucn I at doses of 5, 10 and 20 µg/kg body weight respectively for 42 days. Rats were weighed. Serum testosterone and levels of Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) were measured, in addition to epididymal sperm count, sperm motility and testicular weight. Administration of Ucn I in dose dependant manner induced significant reducing effects upon body weight, testes weight, epididymal sperm count, sperm motility and serum testosterone levels of normal adult rats. Ucn I may possibly affect male infertility by induction of a significant decrease in epididymal sperm count, motility and serum testosterone levels that might be due to a direct effect of Ucn I on testicular tissue and the germ cells without direct involvement of hypothalamic–pituitary–gonadal axis.

Key words: Urocortin I, epididymal sperm count, sperm motility, testosterone, male rat.

INTRODUCTION

Urocortin (Ucn) I, II and III and corticotropin releasing hormone (CRH) are peptide hormones that belong to the

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corticotropin releasing hormone family of peptides. Ucn I has a high affinity for CRF_1 and CRF_2 receptors. However, Ucn II and Ucn III show selective CRF_2 affinity (Hsu and Hsueh, 2001, Karteris et al., 2004).

Ucn I, II and III have been detected in the central nervous system (Bittencourt et al., 1999) as well as in many peripheral tissues (Kageyama et al., 1999; Lewis et al., 2001, Oki and Sasano, 2004). Ucns have roles in diverse physiologic processes such as regulation of the cardiovascular, gastrointestinal, reproductive, immune functions, body weight, food intake, and behavioral responses to stressors (Rodriguez et al., 1983, Fekete and Zorrilla, 2007).

Ucn I is composed of 40 amino acids with 45% sequence identity to CRF and 63% sequence identity to fish urotensin. It was first isolated from rat midbrain (Vaughan et al., 1995; Zhao et al., 1998). The major site of brain Ucn I synthesis is the Edinger-Westphal nucleus (Bittencourt et al., 1999).

The reproductive function of Ucns in male is still unclear. However, both Ucn mRNA and peptide were expressed in mature spermatozoa. Moreover, the expressions of CRF_1 and CRF_2 receptors in spermatocytes and spermatogonia respectively suggested that Ucns may play a role in the germ cell division, differentiation and spermatogenesis (Tao et al., 2007; Rivier, 2008).

Ucn I gene is located in rat testis with lower detectable levels of Ucn II and III mRNA gene expressions indicating the predominance of Ucn I signals. Ucn I gene expression appeared restricted to interstitial Leydig cells (Lee et al., 2011). Hence, the testis is a main target for Ucn I interactions. The prostate is a main source of local Ucn I. The secreted Ucn in the seminal fluid could activate the myometrial contractility in the female genital tract. So, it is suggested that Ucn I may participate in the physiology of fertilization, pregnancy and parturition (Petraglia et al., 1999; Yutaka and Hironobu., 2004, Lee et al., 2011).

Therefore, this study was designed to find out the endocrinal and reproductive function of Ucn I in male through evaluating the effects of exogenous Ucn I on epididymal sperm count, motility and sex hormone profiles in normal adult rats.

MATERIALS AND METHODS

24 adult albino male rats weighing 175 - 200 gm were obtained from the animal house of Faculty of Veterinary Medicine- Zagazig University. They were put under well controlled light and temperature conditions before experiments in the physiology animal house in Faculty of Medicine - Zagazig University. Animals were not manipulated except for feeding or cleaning of houses and were allowed to eat and drink ad libitum. Animals were handled with principles for the care and use of research as adopted by the National Institutes of Health and the approval from Animal Ethic Committee of the institution (Egypt). After one week of acclimatization, the rats were randomly and equally divided into four groups. Group (I): Vehicle-treated group in which control rats received 0.1 mL of normal saline for 42 days. Rats in group II were divided into three equal subgroups. Group (IIA), (IIB) and (IIC): Ucn I-treated groups in which rats were given daily intraperitoneal injections of rat Ucn I at doses of 5, 10 and 20 µg / kg body weight respectively for 42 days (Haron et al., 2010). Rat Ucn I (1 mg, powder form, Sigma Chemical Co., St. Louis, USA) was dissolved with 1% acetate solution and frozen at -80°C until use (Kihara et al., 2001). All injections were performed intraperitoneally in a volume of 0.1 ml/rat.

Samples collection

24 h after the last injection of Ucn I, blood samples were taken from retro-orbital venous plexus. Serum was separated by centrifugation of blood at 3000 rpm for 20 minutes and kept deep frozen at (-20°C). Serum Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) and testosterone levels were measured. Under mild ether anesthesia, the animals were sacrificed by cervical dislocation. Testes were weighed and epididymis was used for the evaluation of sperm parameters. Repeated freezing and thawing of samples were avoided.

Hormonal assays

Serum FSH levels were measured using FSH enzyme immunoassay test kits (BioCheck, CA 94404) according to the method described by Rebar et al. (1982). Both serum LH levels and testosterone levels were measured using LH enzyme immunoassay test kits and testosterone enzyme immunoassay test kits respectively (BioCheck, CA 94404) (Tietz, 1995). All are measured by spectrophotometer (spectronic 3000 Array, Germany) at 450 nm.

Sperm count and motility analysis

For each rat, the right epididymis was dissected, removed and minced in 2 ml of Hank's buffer salt solution (HBSS) (Sigma-Aldrich Co.-USA) at 37°C (Idris et al., 2012). The cauda epididymis sperm was determined using the standard hemocytometer method after 5 min incubation at 37°C, then the epididymal fluid was drawn up to the 0.5 mark of pipette White Blood Cell (WBC) and the semen diluting fluid (sodium bicarbonate 5 g, formalin 1 ml, distilled water 99.0 ml) was drawn up to '11' mark, and subsequently mixed well. One drop was kept in haemocytometer chamber in humid place for 1 h. Then, under the light microscope, the number of spermatozoa in the appropriate haemocytometer squares was counted according to the formula; sperm count =No. of spermatozoa counted x dilution factor x volume factor/ No. of areas counted (Belsey et al., 1980), the sperm concentration refers to the number of spermatozoa / ml fluid was gauged.

Using the number of live sperm cells over the total number of sperm cells (either motile which displayed some movement or non-motile that not move at all), the sperm motility percentage was calculated (Khaki et al., 2009).

Statistical analysis

The statistical analysis of results was done by using Statistical Package for Social Science (SPSS) program, version 18, for windows XP professional. The biochemical data were expressed as Mean ± SD. Statistical analysis were performed using one way analysis of variance (ANOVA) followed by post-hoc multiple comparisons (Tukey test). P value < 0.05 was considered

Table 1. Serum LH, FSH, T levels in Ucn I-treated rats and controls after a duration of 42 days.

Variable	FSH (µUL/mL)	LH (µUL/mL)	T (ng/mL)
Group I (Control rats)	0.43±0.04	0.41±0.07	4.39±0.79
Group IIA	0.40±0.03	0.39±0.05	3.5±0.47
Group IIB	0.38±0.04	0.40±0.04	$2.7±0.34^{p1,p2}$
Group IIC	0.37±0.04	0.37±0.073	$1.87±0.41^{p1,\ p2}$

p1 = significant difference when compared with negative control group, p2 significant when compared with group IIa, p3 when compared with group IIb

Table 2. Epididymal sperm count, motility and testis weight in Ucn I-treated rats and controls after a duration of 42 days.

Variable	Epididymal sperm count (millions/ml)	Total sperm motility (%)	Progressive motility (%)	Testis weight (g)
Group I (Control rats)	53.1±5.9	85.9±9.8	78.9±8.86	1.84±0.3
Group IIA	$43.7±5.5^{p1}$	$70.3±7.4^{p1}$	$61.72±8.1^{p1}$	$1.48±0.17^{p1}$
Group IIB	$40.7±5.5^{p1}$	$67.1±10.6^{p1}$	$59.4±11.\ 1^{p1}$	$1.17±0.16^{p1}$
Group IIC	$35.2±6.1^{p1}$	$60.1±9.2^{p1}$	$56.31±11.35^{p1}$	$0.87\ ±0.13^{p1}$

p1 = significant difference when compared with negative control group, p2 significant when compared with group IIa, p3 when compared with group IIb
Total Motility = Progressive Motility + Non-progressive Motility; Progressive motility: sperms moving actively, either linearly or in large circle regardless of speed; Non-progressive motility: all other motility patterns with an absence of progression (i.e. swimming in a small circle or when only a flagellar beat can be observed); Immotile sperms: no movement.

statistically significant at confidence interval 95 %.

RESULTS

Hormone assays

Table 1 shows the effects of Ucn I on hormones in the studied groups. Following treatment with different doses of Ucn I, the results showed no significant differences were evident in serum FSH and LH levels between rats given Ucn I and their age-matched controls (p1>0.05). However, T was significantly lower in rats treated with 10 and 20 µg / kg (p2<0.04, p2<0.001 respectively) compared to those treated by 5 µg / kg of Ucn I in same group and their age-matched controls (p1 <0.001, p1 <0.001 respectively). As presented in Table 2 Epididymal sperm count, motility and testis weight were significantly lower in rats given Ucn I at doses of 5 µg (p1<0.05, p1<0.05, p1<0.04 respectively), 10 µg / kg body weight (p1<0.006, p1<0.01, p1<0.001 respectively) and 20 µg / kg body weight (p1<0.001, p1<0.001, p1<0.001 respectively) compared to their age-matched controls.

DISCUSSION

Male infertility is a major health problem that represents approximately 30% of all infertilities (Carlsen et al., 1992; Isidori et al., 2006). While stress activates the hypothalamic–pituitary–adrenal (HPA) axis, it suppresses the hypothalamic–pituitary–gonadal (HPG) axis (Kageyama, 2013, Bhongade et al., 2015). Stress profoundly inhibits the reproductive function by suppressing the pulsatile release of hypothalamic gonadotropin-releasing hormone (GnRH) and consequently luteinizing hormone (LH), at least in part via the corticotrophin-releasing factor (CRF) system as well as through the GABAergic system (Lin et al., 2012, Bhongade et al., 2015).

The release of CRF in response to various stressors suppresses of the HPG axis, especially the GnRH pulse generator in the hypothalamus, and also decreases GnRH mRNA levels via the CRF_1 receptors (Kagayama, 2013). Locally, CRF was found to exert an inhibitory effect on Leydig cell activity in rat testes (Dufau et al., 1993). However, Urocortin I (Ucn I), rather than CRF, is located in rat Leydig cells (Hardy et al., 2005).

Ucn I is an endogenously secreted corticotrophin-releasing factor (CRF)-related peptide. Ucn I is a 40-amino acid peptide that shares 45% homology with CRF (Vaughan et al., 1995; Zhao et al., 1998). In addition, Ucn I exerts its biological activity through CRF_1 and CRF_2 receptors and binds to both types with high affinity (Hsu and Hsueh, 2001). Neurons in the centrally projecting Edinger-Westphal nucleus are the main site of Ucn I synthesis in the mammalian brain, and are assumed to play a role in the stress response. Acute and chronic stress resulted in an increase in Ucn I content of the

Edinger-Westphal nucleus (Derks et al., 2012).

Both CRF and Ucn I contribute to stress responses, cardiovascular and gonadal functions via G protein-coupled seven transmembrane receptors (Vale et al., 1997; Kageyama et al., 1999a; Suda et al., 2004). It is well known that anxiety has a detrimental effect on fertility (Demyttenaere et al., 1988). Ucn I was found to elicit an increase in anxiogenic behavior and potentiate the anxiogenic action of ghrelin as well (Currie et al., 2014).

Therefore, this study is implemented to find out the probable endocrine and reproductive actions of Ucn I in adult male rats via investigation of the effects of exogenous Ucn I on epididymal sperm count, motility, testicular weight and sex hormone profiles. The study of the current hypothesis is that CRF-related peptides act within the gonads, rather than in the periphery, and that their influence on Leydig cells activity is at least partly due to rapid decreases in levels of the steroidogenic enzymes (Herman and Rivier, 2006).

In the present study, Ucn I administration for 42 days significantly decreased the testis weight, epididymal sperm count and motility. These findings are collaborated by the findings of Tao et al. (2007) who hold that Ucn I significantly inhibited the sperm motility and ascrosome reaction in a concentration-dependent manner. It inhibited T-type calcium channels in mouse spermatogenic cells, sperm motility and progesterone-evoked sperm acrosome reaction, indicating that inhibition of Ca^{2+} channels may be a mechanism for the inhibitory effects of Ucn I on male reproductive functions. Ucn I might decrease Ca^{2+} via inhibiting T-type calcium channels directly in male reproductive cells, instead of binding to its receptors firstly (Tao et al., 2005).

The duration of treatment was documented by Haron et al. (2010). As the germ cells are arranged in specific cell associations, called the stages of the cycle of the seminiferous epithelium. For the rat, the fourteen different stages of each cycle of the seminiferous epithelium last approximately 13 days. The germ cell traverses the different stages of the cycle four times for its complete development, which takes all together 52 days (Clermont, 1972; Karl et al., 1991).

Intraperitoneal administration of Ucn I in the present study significantly decreased the serum testosterone levels. These results are in consistent with findings of Rivier (2008) who reported that intratesticular administration of CRF and Ucn I significantly inhibited the testosterone response to LH-like molecules such as human chorionic gonadotropin (hCG). Ucn I was more effective than CRF in inhibiting Leydig cell responsiveness.

This study further suggested that the intraperitoneal Ucn I administration may be an effective route in inhibition of the release of testosterone from Leydig cells. Ucn I may penetrate the testes after its intraperitoneal injection and acted via a testicularly mediated site of action. Blood-borne compounds readily penetrate the intensely vascularized testes.

In the present study, no significant differences were evident in serum FSH and LH levels between Ucn I-treated rats and their age-matched controls. These results are in agreement with findings of Rivier (2008) who reported that blockade of endogenous LH before Ucn I injection did not alter the inhibitory effect of this peptide regardless of whether it was administered into the general circulation or into the testes.

In addition, it was suggested that the inhibitory influence of CRF-related peptides on testosterone response to gonadotropins is primarily exerted through CRF_1 receptor activation (Li et al., 2005). It is Ucn I, rather than CRF, that is located in rat Leydig cells (Hardy et al., 2005). However, stress-induced suppression of LH pulses was mediated by CRF_2 receptors probably through pituitary Ucn II (Nemoto et al., 2010).

Conclusion

The results of this study indicate that Ucn I administration significantly reduces testis weight, sperm count, motility and serum testosterone level which does not seem to directly involve the hypothalamic–pituitary–gonadal axis but might be due to a direct effect of Ucn I on testicular tissue and the germ cells.

Conflict of Interests

The authors have not declared any conflict of interests.

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