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Full Length Research Paper

Scrotal circumference and body measurements of Yankasa rams following exposure to Cypermethrin

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This work was designed to access reproductive status of Cypermethrin treated rams by measuring the scrotal circumference, live weight, live testicular width and length as well as some physiological parameters to determine its reproductive and general toxicity in Yankasa rams. Sixteen rams aged 18 to 30 months and weighing between 21.5 and 46.5 kg were used for this study. The 16 rams were divided equally into two groups: A (treatment) and B (control). Group A were given Cypermethrin (3%) at the dose rate of 3 mg/kg (0.1 ml/kg) body weight, topically. While group (B) were given distilled water at the same dose rate and route. These treatments were repeated fortnightly for a period of 12 weeks. The animals were weighed weekly using a measuring scale early in the morning before feeding. Their rectal temperature and respiratory rates were taken concurrently. Scrotal circumference, testicular width and length were measured weekly with the use of flexible measuring tape. Results showed no statistically significant difference between the two groups in all the parameters measured (P>0.05). It was concluded that Cypermethrin at the dose rate of 3 mg/kg body weight for twelve weeks increased body weight and scrotal circumference of treated rams, although there was no significant difference between the treated and control rams P>0.05. The treatment did not have any effect on the live testicular width and length, body temperature as well as respiratory rate of Yankasa rams studied. It was recommended that similar studies should be conducted in other domestic ruminants because species differences may play key roles in reproductive and general toxicity of Cypermethrin.

Key words: Scrotal circumference, Cypermethrin, measurements, rams, toxicity.

INTRODUCTION

Pyrethroids have been known as being a potential endocrine disruption compound through human and other mammalian studies. These studies vary in compounds, dosages, routes, exposure durations and evaluated parameters (Martenies and Perry, 2013). Other human research with pesticide endocrine disruption on male reproductive performance consistently focused on concentration, motility and morphology based on World Health Organization (WHO) fertility parameters (Martenies and Perry, 2013). Decreased sperm concentrations were most commonly reported with decreased motility being less frequent and morphology

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being less clear clinically (Martenies and Perry, 2013).

The absorption and elimination of Cypermethrin is reported as rapid in the different mammalian species tested (WHO, 1989b). The half-life in the fat of rats is about 9 - 12 days for the cis-isomer and 3-4 days for the trans-isomer, the acute toxicity of Cypermethrin for mammals is of moderate order. The oral LD_{50} for the rat ranged from 200 - 400 mg/kg body weight, short term and long-term toxicity studies on rats, mice, and dogs have shown effects on growth, liver, kidneys, nervous system and blood (WHO, 1989b).

Cypermethrin is toxic not only to insects but also mammals (Barlow et al., 2001; He, 2000). Clinical signs like muscular tremors, ataxia, weakness of limbs, convulsions, coma and death from respiratory depression have been reported in animals after ingesting high doses of Cypermethrin (Sandhu and Brar, 2000). Incoordination, muscular tremors, jerky movements, ataxia, staggering gait and dizziness were observed in dose dependent manner in Cypermethrin treated rabbits (Ullah et al., 2006). Long-term feeding studies with laboratory animals have shown that Cypermethrin causes adverse effects. In rats, it caused reduced growth rate and increased liver weight. In mice, it caused reduced weight gain, mild anemia, and increased liver weight. In dogs, it caused loss of appetite, incoordination and tremors. In rabbits, it caused pathological changes in the thymus, liver, adrenal glands, lungs and skin (Caroline, 1996).

Besides generalized toxic effects of Cypermethrin, decreased number of implantation sites, number of viable fetuses and weight gain of fetuses in rabbits treated with Cypermethrin have been reported (Elbetieha et al., 2001). Histologically, changes were observed in ovary and uterine tissues which were more pronounced at higher doses (EL-Toukhy and Girgis, 1993). The effect of pyrethroid exposure in female animals has been well elaborated by many researchers. (Liu et al., 2011; Petr et al., 2013; Gill et al., 2011; Lemos et al., 2011; Guerra et al., 2011).

Cypermethrin is able to influence some reproductive and fertility parameters as exposure to this chemical can cause significant increase in the production of non-viable or abnormal sperm in mice (Jalal et al., 2010). High oral dosages (30 to 60 mg/kg) of Cypermethrin on adult male rats for 15 days decrease daily sperm output (Yan et al., 2013). High dosages of Cypermethrin also caused histological atrophy and distortion of seminiferous tubules including deformed and disordered arrangement of germ cells. Vacuolization of Sertoli cells and deforming of Leydig nuclei was also noted with subsequent decrease of serum testosterone concentration in oral Cypermethrin treated rats (Yan et al., 2013). Oral treatment of 5 mg/kg deltamethrin for 4 weeks decreased testosterone, LH and FSH concentrations (Ismail and Mohamed, 2012). Male ruminants exposed to pyrethroids have also been clinically observed to have negative effects on reproduction. Observations by Volkmann and Voelkl (2012) implied that pyrethroid exposure to bulls and rams for a short duration could negatively impact sperm concentration, ejaculate volume, progressive sperm motility and sperm morphology. Recently, Cain et al. (2014) showed no differences in sperm motility or morphology throughout an 84-day trial period with exposure to 150% label dose, giving twice (day 0 and 14), of 1% Permethrin dermally to purebred beef bulls. French et al. (2014) found no effect on sperm parameters when crossbred bulls were exposed to Cyfluthrin pour-on, Cyfluthrin fly tags and a combination of Cyfluthrin pour-on and fly tags over a nine week study. Likewise, crossbred bulls exposed to different combinations of pyrethroids 28 and pyrethrins (control – cyfluthrin pour-on and fly tags, treatment – cyfluthrin pour-on and fly tags and pyrethrin premise spray fogger) showed no consistent change in sperm parameters or testosterone in a nine week study (Stewart et al., 2015). Ingestion of Cypermethrin at high doses (18.93 or 39.66 mg per day) resulted in a significant increase in the weights of testes and seminal vesicles of male Sprague Dawley rats, also epididymal and testicular sperm counts as well as daily sperm production were significantly decreased in exposed males (Jalal et al., 2010). Popular press literature has identified potential links between use of pyrethroids and its negative effects on beef bull reproductive health (Ismail and Mohamed, 2012).

The mechanism by which Cypermethrin affects male reproduction is unclear (Wang et al., 2009). Pyrethroids are rapidly metabolized in mammals and several studies have shown that Cypermethrin damages the brain, liver and erythrocytes by causing oxidative stress (Wang et al., 2009). Three doses of β-Cypermethrin decreased body weight gain and weight of testosterone-sensitive organs such as testes, epididymis, seminal vesicles and prostate glands, sperm count, viability and intact acrosome population (Wang et al., 2009). Qualitative analyses revealed that low dose (1 mg/kg) of beta-Cypermethrin decreased the number of interstitial Leydig cells but did not affect the intratubular compartment of seminiferous tubules, as the concentration of beta-Cypermethrin increased, the number of spermatids and cells in the seminiferous tubules decreased (Wang et al., 2009). Low dose of beta-Cypermethrin did not significantly affect sperm concentration, while a high dose (20 mg/kg) significantly reduced the number of sperm cells in the seminiferous tubules, serum testosterone and steroidogenesis acute regulatory protein (StAR) (Wang et al., 2009). Experiment on the effect on biochemical parameters of testes after administration of 250mg/kg, per os of α- Cypermethrin in albino mice was reported to cause histologic changes in spermatogenic cells, like rupture of cell membrane, shrinkage in the nucleus, presence of stages of apoptosis, condensation of chromatin and decrease or absence of cytoplasmic organelles, as revealed by transmission electron microscopy (TEM) (Prakash, 2010). Decrease in food

intake, body weight, absolute and relative gonad weights have been observed in rabbits treated with Cypermethrine (Handerson and Parkinson, 1981).

Pesticide exposure is associated with infertility, there is much concern that exposure to environmental contaminants causes decreased sperm counts, impairment of sperm motility, reduced fertilization ability, producing abnormal sperm in men and wildlife (El-betieha et al., 2001). A significant decrease in testicular index weight, sperm mass motility and spermatozoa concentration in the epididymis was also reported following oral administration of Cypermethrin (Assayed et al., 2008). The cytotoxic action has been suggested to be associated with a decrease in testicular index weight of male rats. Such decrease could also be linked to the reduced testosterone synthesis and disruption of normal androgen status (Abd-Allah, 1995). Effects of pyrethroid exposure have been assessed with standard breeding soundness examination (BSE) parameters and steroid (testosterone) concentrations; however, effect of pyrethroids on testicular histopathology has not been addressed in the bovine (Tyler, 2015). Morphology difference after dermal exposure to pyrethroids was not significantly different as compared to controls in all previously published bull data (Cain et al., 2014; French et al., 2014). Given that a BSE is a “snap shot” evaluation of bull reproductive soundness, further research may be warranted to elucidate the long-term effects of chronic pyrethroid exposure. There were no differences noted in average daily gain or body condition scores due to treatment, which was expected due to the short duration of only 19 days between initial and final body weight (BW) and body condition score (BCS) (Tyler, 2015). Scrotal circumference and testicular measurements are component parts of breeding soundness examination in the ram. This work aimed to access reproductive status of treated rams by measuring the scrotal circumference, live weight, live testicular width and length as well as some physiological parameters to determine its reproductive and general toxicity in Yankasa rams.

MATERIALS AND METHODS

Study location

The study was carried out at the National Animal Production Research Institute (NAPRI) Shika, Ahmadu Bello University Zaria, which is situated in the Northern Guinea Savannah and lying between latitudes 11° and 12°N and longitude 7° and 8°E, at an elevation of 650 m above sea level. The area has an annual rainfall of 1100 mm (Igono et al., 1982). There are two seasons {rainy season (May-October) and dry season (November-April)}, respectively.

Experimental animals

The animal experiments followed the principles of the laboratory animal care (Canadian Council on Animal Care Guide, 1993). Sixteen sexually-mature, healthy Yankasa rams aged 18 to 30 months, weighing between 21.5 and 46.5 kg with clinically normal genitalia were used in this study. The rams were purchased from the open market in Sabua Local Government Area of Katsina State. They were housed at the Small Ruminant Research Programme Experimental Unit of NAPRI. The house was made of brick concrete pens with concrete floors. The rams were divided into two groups of eight each. They were given concentrate feed *ad libitum* (cotton seed, maize offal, maize, wheat offal, bone meal and salt) in the morning and later in the evening; hay was made available during the day at intervals. The hay used was *Digitaria smutsii*, and water was given *ad libitum*.

Experimental design and treatment

The 16 rams were divided equally into two groups (A and B). Group A served as the treatment group, while group B served as the control. The animals were acclimatized for two weeks during which blood and fecal samples were collected and analyzed for haemoparasites and helminths and treatments where given when necessary.

Administration of 3% cypermethrin

The rams in group A were given Cypermethrin (3%) at the dose rate of 3 mg/kg (0.1 ml/kg) body weight, topically as pour-on. The control group B rams were given distilled water at the same dose rate of 0.1 ml/kg body weight topically as pour-on. These treatments were repeated every two weeks for a period of 12 weeks. Body and testicular measurements, scrotal circumference, body temperature, respiratory rate were collected before the administration of 3% Cypermethrin to establish base line data. This was done by obtaining the average values for the eight animals in each group before the experiment started.

Scrotal circumference and body measurements

The animals were weighed weekly using a measuring scale (Salter suspended weigher, model 235, UK) early in the morning before feeding. Their rectal temperatures (using a clinical thermometer) and respiratory rates (using stop watch) were taken concurrently. Scrotal circumference, testicular width and length were measured weekly with the use of flexible measuring tape.

Statistical analysis of data

Data were expressed as means and standard error of mean (SEM). Data were analyzed using descriptive statistics and paired student’s t-test with SPSS/PC computer program (Version 20.0, SPSS®, Chicago IL, USA). Differences with confidence values of $p < 0.05$ were considered statistically significant (Daniel, 1991).

RESULTS AND DISCUSSION

The baseline parameters (Table 1) for the two groups were within normal physiological range for rams.

Body weights and scrotal measurements

The mean weekly body weights of the treated and the control groups are presented in Table 2. There was no

Table 1. Base line data for scrotal circumference, body measurements,body temperature and respiratory rate in both the treated and control rams (Mean ± SEM).

Parameters	Treated (n=8)	Control (n=8)
Body weight (kg)	31.75 ± 3.11	32.20 ± 1.81
Scrotal circum. (cm)	26.75 ± 1.20	26.25 ± 0.70
Testicular length (cm)	15.19± 0.35	15.13 ± 0.49
Testicular width (cm)	7.00 ± 7.00	6.19 ± 0.45
Body temperature (°C)	39.26 ± 0.10	39.29 ± 0.14
Respiratory rate (r/min)	52.00 ± 7.60	52.50 ± 4.10

Table 2. Mean weekly body weight (kg) and scrotal circumference (cm) of Yankasa rams during the treatment period (Mean ± SEM) n=8.

Weeks	Body weight (kg)		scrotal circumference (cm)	
	Treated	Control	Treated	Control
1	32.59± 1.86	30.85± 3.20	25.81 ± 0.77	24.69 ± 1.31
2	32.50 ± 2.09	31.06 ± 3.16	24.88 ± 0.78	24.06 ± 1.23
3	32.06 ± 2.52	30.94± 3.17	24.38 ± 0.94	23.94 ± 1.19
4	31.25 ± 2.54	30.87 ± 3.21	24.81 ± 1.06	23.88 ± 1.07
5	31.38 ± 2.60	30.35 ± 3.28	24.81 ± 1.26	24.69 ± 1.15
6	31.19 ± 2.75	30.05 ± 3.22	24.75 ± 1.25	24.94 ± 1.21
7	30.91 ± 2.95	30.75± 3.30	24.94 ± 1.25	25.00 ± 1.32
8	31.38 ± 3.02	30.75 ± 3.30	24.69 ± 1.21	24.81 ± 1.33
9	29.94 ± 3.09	29.25 ± 3.24	25.00 ± 1.22	25.19 ± 1.23
10	29.75 ± 2.68	28.63± 3.19	25.88 ± 1.13	24.50 ± 1.08
11	30.75 ± 2.91	29.23 ± 3.34	25.69 ± 1.15	24.81 ± 1.01
12	30.54 ± 2.88	28.13 ± 3.21	25.75 ± 1.16	25.63 ± 1.13

There was no statistically significant difference between the two groups during the treatment period (P>0.05).

statistically significant difference between the two groups during the treatment period (P>0.05). At week 12 of the experiment, the mean body weight of the treated group was higher than the mean body weight of the control group. The difference was not statistically significant (P>0.05). The mean weekly scrotal circumference of the treated and the control groups are presented in Table 2. There was no statistically significant difference between the two groups during the treatment period (P>0.05). At week 12 of the experiment, the mean scrotal circumference of the treated group was higher than the control group and the difference was statistically not significant (P>0.05).

Testicular measurements

The mean weekly testicular length of the treated and control groups are presented in Figure 1. The difference was statistically not significant during the treatment period (P>0.05). The mean weekly testicular width of the treated and control groups are presented in Figure 2. There was no statistically significant difference between the two groups during the treatment period (P>0.05).

Body temperature and respiratory rate

The mean weekly body temperature of the treated and control groups are presented in Figure 3. There was no statistically significant difference between the two groups during the treatment period (P>0.05). The mean weekly respiratory rate of both the treated and control groups are presented in Figure 4. There was no statistically significant difference between the two groups during the treatment period (P>0.05).

DISCUSSION

The results showed that Cypermethrin treated rams lost weight in the first seven weeks of the study and then re-

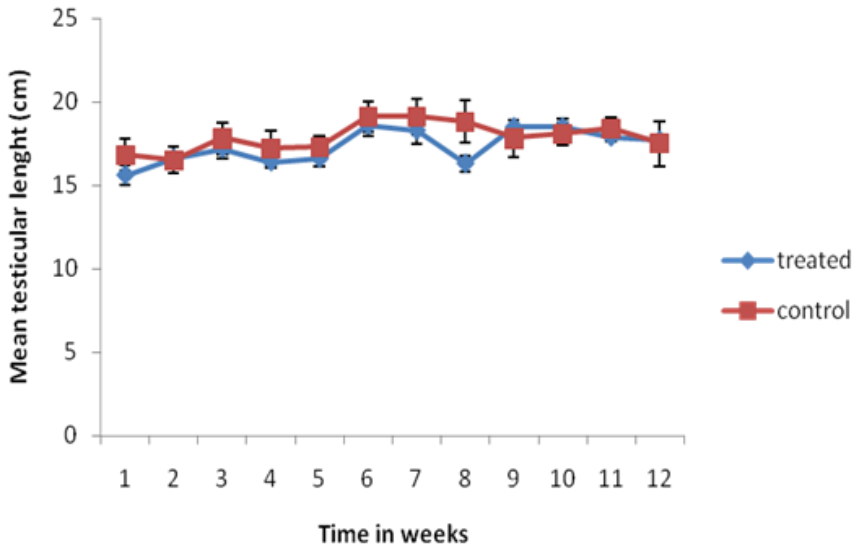

Figure 1. Mean weekly testicular length (cm) of Yankasa rams during the treatment period.

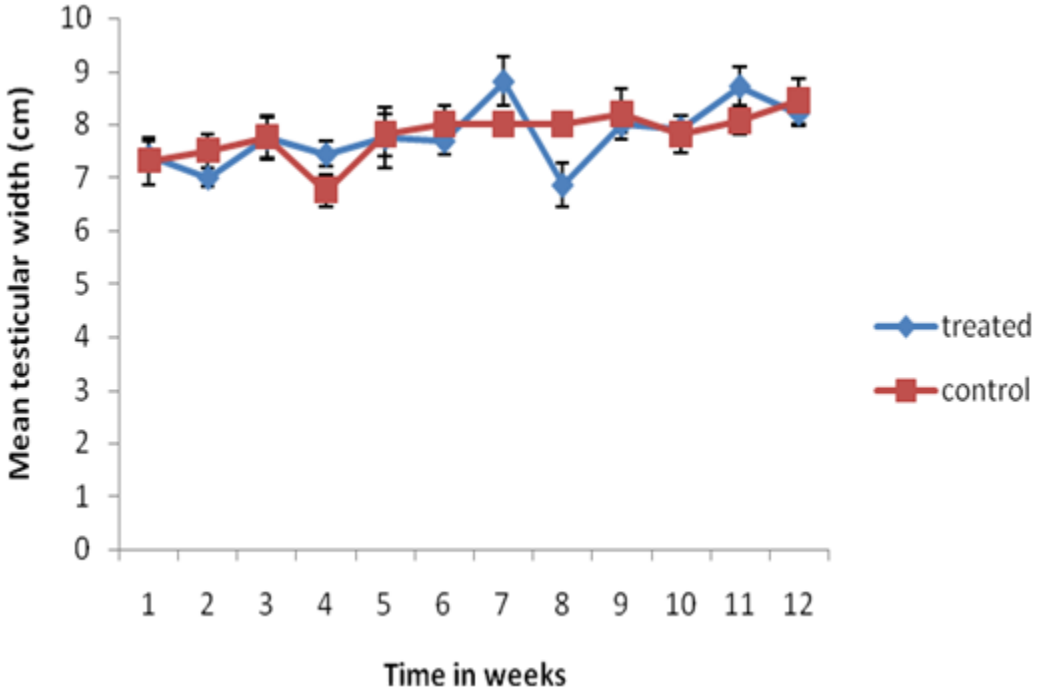

Figure 2. Mean weekly testicular width (cm) of Yankasa rams during the treatment period.

gained weight as from week eight of the study, while the control rams gradually lost weight till week twelve of the experiment. The difference between the live weight of the treated and control rams was not statistically significant during the treatment period (P>0.05). This finding in the first seven weeks of the experiment agrees with the report in mice on long-term feeding studies where Cypermethrin caused reduced weight gain and increased liver weight (Caroline, 1996). Decrease in food intake, body weight, absolute and relative gonad weights have been observed in rabbits treated with Cypermethrine (Handerson and Parkinson, 1981). Three doses (1, 10 and 20 mg/kg)/os of beta- Cypermethrin caused decrease in body weight in male mice (Wang et al.,

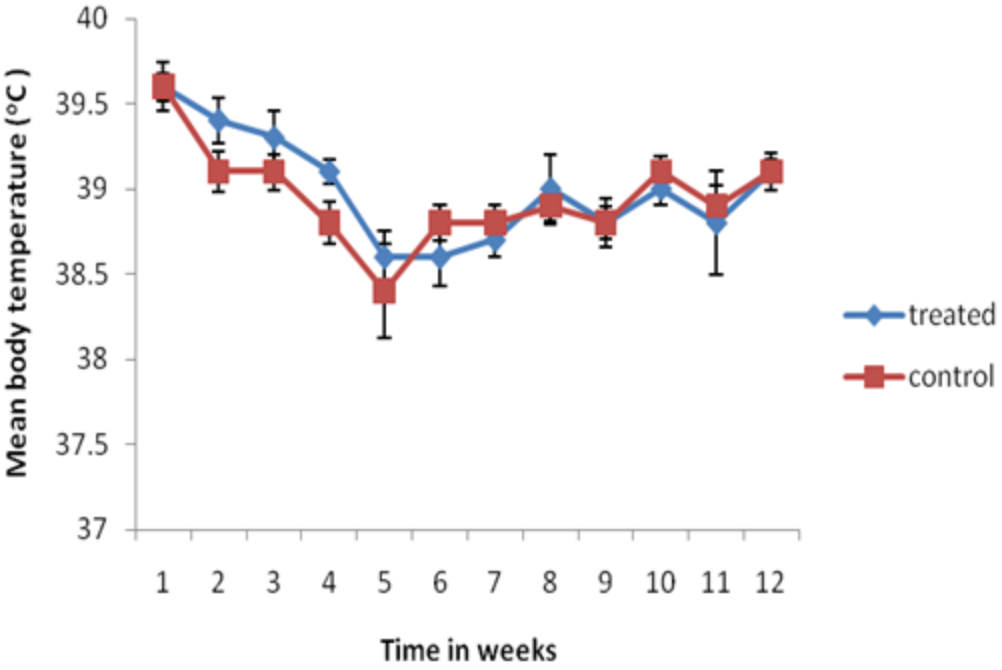

Figure 3. Mean weekly body temperature (°C) of Yankasa rams during the treatment period.

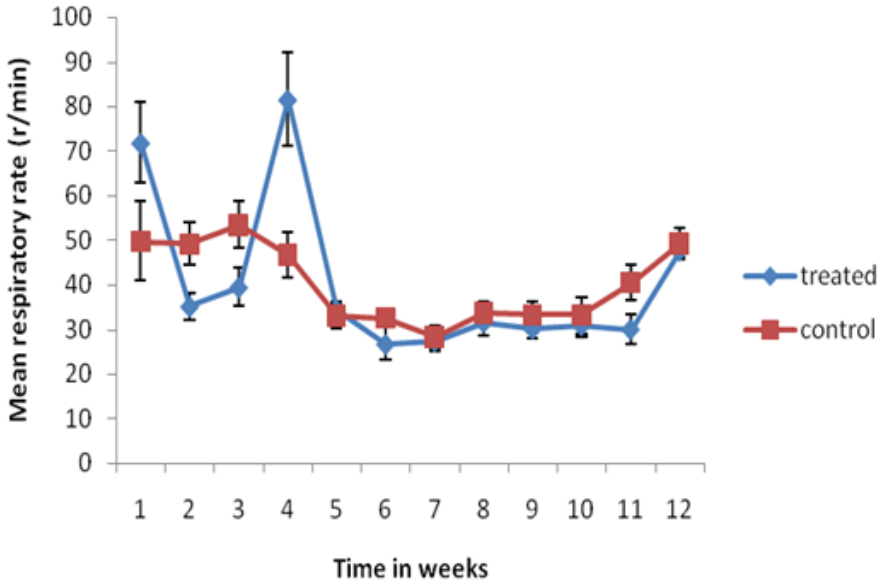

Figure 4. Mean weekly respiratory rate (r/min) of Yankasa rams during the treatment period.

2009).

However, the gain in weight of the treated rams from weeks eight to twelve contradicts the above report. The contradiction may have resulted from the route of administration and the species of animals involved in the study. The gain in weight may be due to the effect of Cypermethrin on body fat as Cypermethrin is lipotropic. The highest level of Cypermetherin is found in body fat, which is consistent with lipophilic nature of the compound (WHO, 1989a). There may be more fat depots e.g. the subcutaneous fat in rams where Cypermethrin exerted its effect just like in the liver of rats and mice which

culminated into overall weight gain. It is possible that fly repellant property of Cypermethrin made the treated rams to feed better than the control rams, hence gaining weight over time.

The scrotal circumference of both the treated and control rams followed the same trend with the body weight of the rams. This pattern is expected because scrotal circumference is proportional to the body weight and size. However, there was no statistically significant difference between the scrotal circumference of the treated and control rams during the treatment period. The testicular measures showed that there was a slight increase in the testicular length of both the treated and control groups during the treatment period. But there was no statistically significant difference between the testicular length of the treated and control rams. Similarly, the testicular width of both treated and control rams slightly increased during the treatment period, but there was no statistically significant difference between the testicular width of the treated and control animals during the period. These findings showed that Cypermethrin has no effect on the scrotal circumference, testicular length and width of Yankasa rams. In agreement, Cain et al. (2014) who showed no differences in sperm motility or morphology throughout an 84-day trial period with exposure to 150% label dose, giving twice (day 0 and 14) of 1% Permethrin dermally to purebred beef bulls. It is also in accordance with French et al. (2014) who found no effect on sperm parameters when crossbred bulls were exposed to Cyfluthrin pour-on, Cyfluthrin fly tags, and a combination of Cyfluthrin pour-on and fly tags over a nine weeks study. Likewise, crossbred bulls exposed to different combinations of pyrethroids 28 and pyrethrins (control – cyfluthrin pour-on and fly tags, treatment – cyfluthrin pour-on and fly tags and pyrethrin premise spray fogger) showed no consistent change in sperm parameters or testosterone in a nine weeks study (Stewart et al., 2015). In bovines, there were no differences noted in average daily gain or body condition scores due to treatment, which was expected due to the short duration of only 19 days between initial and final body weight (BW) and body condition score (BCS) (Tyler, 2015). Although, observations by Volkmann and Voelkl (2012) implied that pyrethroid exposure to bulls and rams for a short duration could negatively impact sperm concentration, ejaculate volume, progressive sperm motility and sperm morphology. In the present study, Cypermethrin treated group did not show any significant difference from the testicular length and width of the control group ($P>0.05$). The lack of difference may be due to the dose of 3 mg/kg body weight used in the experiment. The findings showed that Cypermethrin given at the dose of 3 mg/kg body weight to Yankasa rams has no effect on the body temperature of the rams. Again, most reports on the effect of Cypermethrin on health generally were made on laboratory animals. Specific report on the effect of Cypermethrin on the body temperature of rams has not been elaborated. Instead, emphasis was made on its' nervous and general effects on the body. This study also revealed that the respiratory rate of both the treated and control groups reduced over time. But the difference between the treated and control groups during the treatment period was not statistically significant. That is to say that Cypermethrin at the dose rate 3 mg/kg body weight used in this experiment did not affect the respiratory rate. However, coma and death from respiratory depression have been reported in animals after ingesting high doses of Cypermethrin, while its dermal contact in facial area may cause a subjective sensation of tingling or numbness (Sandhu and Brar, 2000).

Conclusion

It was concluded that the use of Cypermethrin topically at the dose rate of 3 mg/kg body weight for twelve weeks is safer than other routes and doses. The treatment did not have any effect on the live testicular width and length, body temperature and respiratory rate of Yankasa rams. Gross observations of fertility parameters in Cypermethrin exposed ruminants like scrotal circumferences may be deceptive. It was recommended that similar studies including histopathology and semen evaluation should be conducted in other breeds and ruminants because species differences and several other factors may play key roles in reproductive and general toxicity of Cypermethrin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of ayurvedic preparation 'Mrityunjay' in digoxin-induced arrhythmic rats

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Mrityunjay is a plant based ayurvedic preparation which is used in the treatment of high blood pressure as well as other cardiovascular diseases. It comprises of significant cardioprotective constituents. This study was conducted to evaluate the effect of Mrityunjay on digoxin-induced arrhythmia and lipid profile in rats. Rats were orally pre-treated with Mrityunjay at the doses of 0.28 and 2.8 mL/kg b.w. for consecutive 35 days through the oral route. On the 36th day, the rats were given a bolus dose of digoxin (20 mg/kg b.w., i.p.). Electrocardiogram along with heart rate were taken for an hour after digoxin administration and serum lipid profile was measured. The digoxin administration caused severe arrhythmia in rats. Mrityunjay significantly (p<0.05) inhibited digoxin-induced arrhythmia at both dose levels. In addition, it caused a significant (p<0.05) decrease in total cholesterol, low-density lipoprotein cholesterol and an increase in high-density lipoprotein cholesterol levels in blood serum. The study revealed that the ayurvedic preparation Mrityunjay possesses significant anti-arrhythmic activity against digoxin-induced arrhythmia. It also has a significant hypocholesterolemic effect.

Key words: Mrityunjay, ayurvedic preparation, cardioprotection, digoxin-induced arrhythmia, electrocardiography (ECG), lipid profile.

INTRODUCTION

Cardiovascular diseases (CVDs) are one of the major causes of mortality in the world (Zheng et al., 2013). As proclaimed by World Health Organization (WHO), CVDs was the leading non-communicable disease (NCD) in 2012 which caused 17.5 million, or 46% of NCD deaths (Mendis et al., 2015). In recent times, WHO are giving emphasis on the concomitant use of traditional formulations, which are largely based on plant materials, to ensure total health coverage among herbal remedies; use of the ayurvedic system of medicine is prevalent in Bangladesh. Patients due to their poor economic condition depend on cost effective and affordable herbal drugs that are being used traditionally for centuries (Vogel et al., 2005). Ayurvedic products may exert

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significant therapeutic effects on the cardiovascular system (Lodha and Bagga, 2000). It has been found that there is a large demand for ayurvedic drugs for the remedies of CVDs. However, clinical applications of ayurvedic preparations are being thwarted due to their lack of quality, toxicological and pharmacological evidence (Chandra, 2016). Hence, WHO has emphasized for the evaluation of quality, safety and therapeutic efficacy of ayurvedic preparations (Chaudhary and Singh, 2001).

Mrityunjay is a plant-based ayurvedic preparation which is used for the treatment of high blood pressure as well as other CVDs, according to Bangladesh National Formulary of Ayurvedic Medicine. The preparation contains root extracts of *Rauwolfia serpentina* L. (family: Apocynaceae), *Withania somnifera* L. (Solanaceae), *Glycyrrhiza glabra* L. (Fabaceae), *Glycyrrhiza glabra* L. (Fabaceae), *Acorus calamus L.* (Acoraceae), *Roscoea purpurea* Sm. (Zingiberaceae), bark extract of *Terminalia arjuna* Roxb. (Combretaceae), fruit extracts of *Terminalia chebula* Retz., *Terminalia bellirica* Roxb. (Combretaceae), *Phyllanthus emblica* L. (Phyllanthaceae), wood extract of *Acacia catechu* L.f. (Mimosaceae), a whole plant extract of *Tragia involucrate* L. (Euphorbiaceae), spikenard of *Nardostachys jatamansi* (Caprifoliaceae) and Jaggery (Anonymous, 1992).

R. serpentina is a medicinally important herb which is extensively used in the treatment of hypertension and psychotic disorders including anxiety, insanity, insomnia, schizophrenia, etc (Qureshi and Udani, 2009; Mashour et al., 1998). *Invivo* study revealed that methanol extract of *R. serpentina* may exert significant hypolipidemic and hypoglycemic activities (Qureshi and Udani, 2009). The main compound of *R. serpentina*, ajmaline, has been found to alleviate digoxin toxicity (Obayashi et al., 1976). The cardiotonic effect of the plant *T. arjuna* is evident in the ayurveda (Tripathi et al., 1996). The bark of the plant is used as anti-ischemic and cardioprotective (Bone and Morgan, 1996). *T. arjuna* bark extract has been found to be beneficial for heart failure, coronary artery disease, and high cholesterol levels coronary arterial disease (Subramaniam et al., 2011; Kapoor, 2001). It has been found to significantly increase the activity of lipoprotein lipase as well as plasma lecithin-cholesterol acyltransferase mediated hepatic bile acid synthesis and reduces the activity of lipogenic enzymes including 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA) reductase, glucose-6-phosphate dehydrogenase and malate dehydrogenase (Patil et al., 2011). The root extracts of *W. somnifera*, *A. calamus*, *R. purpurea* and *G. glabra* have been found to possess significant hypocholesterolemic effect (Bathla et al., 2016; Visavadiya and Narasimhacharya, 2007; Visavadiya and Narasimhacharya, 2006; Parab and Mengi, 2002). Oral administration of the fruit extracts of *T. chebula*, *T. bellirica* and *P. emblica* have been found to cause a significant reduction in serum cholesterol level (Maruthappan and Shree, 2010; Latha and Daisy, 2010; Mathur et al., 1996). The hardwood extract of *A. catechu* exhibited promising anti-dyslipidemic activity (Srivastava et al., 2011). Spikenard possesses significant antiarrhythmic and hypotensive activities (Disket et al., 2012). Jaggery has been found to significantly reduce the degree of atherosclerosis (Okabe et al., 2009).

Although Mrityunjay possesses significant cardio-protective ingredients, there is a paucity of scientific evidence regarding its cardiac effects. Therefore, considering the significance of the evaluation of safety and efficacy of ayurvedic drugs, therapeutic implication of Mrityunjay in ayurveda and promising cardioprotective properties of its ingredients, the present study was carried out to investigate its cardioprotective effects in an experimental animal model. Cardioprotective effect of the ayurvedic preparation was evaluated by the means of its anti-arrhythmic (against digoxin-induced arrhythmia) and hypocholesterolemic activities, along with its effect on heart rate, in a rat model.

MATERIALS AND METHODS

Drugs used in the study

The ayurvedic preparation, Mrityunjay, was procured from *Sree Kundeswari Aushadhalaya* Ltd., Batch#003), Chittagong, Bangladesh. As described on the manufacturer's label, each 5 mL of the preparation contains 28.24 mg of the extract of *R. serpentine* root, *T. arjuna bark, G. glabra* root*, T. involucrate* whole plant and other ingredients individually according to the Bangladesh National Formulary of ayurvedic Medicine. Ketamine hydrochloride (Gonoshasthaya Pharmaceuticals Limited, Dhaka, Bangladesh) and digoxin (Aristopharma Ltd., Dhaka, Bangladesh) were obtained as gifted samples. Diagnostic kits were purchased from Exim GmbH (Germany) for the measurement of lipid profile.

Experimental animals

Twenty healthy Sprague-Dawley albino rats (90-120 g) were obtained from the animal resources center of the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka. The rats were individually housed in stainless steel cages at room temperature and with sufficient ventilation. The rats of either sex were allowed to acclimatize for seven days prior to drug pre-treatment. They were randomly divided into four groups (n = 5). Distilled water was the only source of fluid, along with liquid drug in pre-treated groups. Each group of rats were provided with sufficient fluid and feeds and re-weighed after 35 days of pre-treatment.

Experiment protocol

Animals were grouped into four groups containing 5 animals in each group (n = 5). Group one rats was given normal food and water *ad libitum* thrice daily for 35 days. This group of rats was referred to as the control rats. Group two rats were given food and water *ad libitum* thrice daily for 35 days. On the 36thday, digoxin was administered (20 mg/kg b.w., i.p.). This group of rats is referred to as digoxin control rats. Group three rats were given normal food and water *ad libitum* with Mrityunjay low dose (0.28 mL/kg b.w.) orally (p.o.) for 35 days. On the 36th day, digoxin was administered (20 mg/kg b.w., i.p.); this group is referred to as the Mrityunjay low dose pre-treated rats. Group four rats were given normal food and

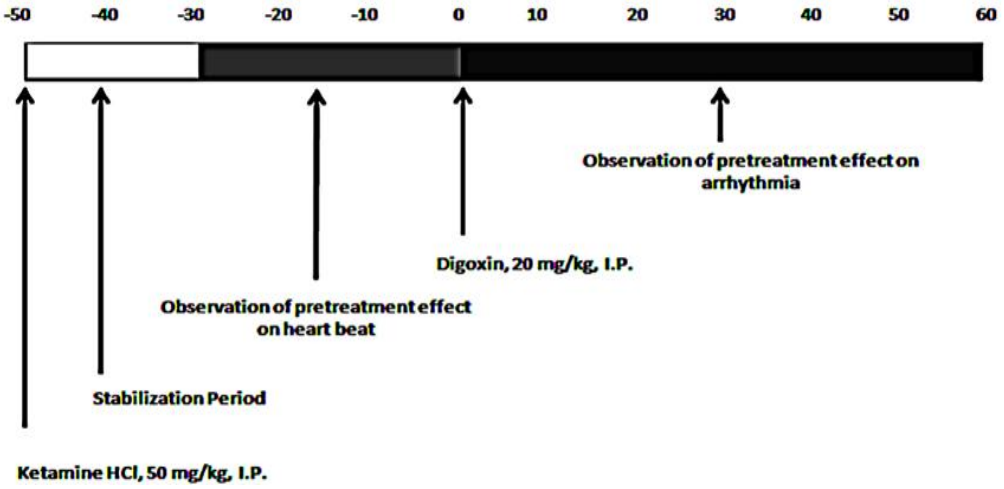

Figure 1. Schematic diagram of the experimental protocol used in the study. The numbers above the bar indicate time (min).

water *ad libitum* with Mrityunjay high dose (2.8 mL/kg b.w.) orally (p.o.) for 35 days. On the 36th day, digoxin was administered (20 mg/kg b.w., i.p.). This group of rats is referred to as the Mrityunjay high dose pre-treated rats. Mrityunjay pre-treatment groups received recommended dose of the drug (10 mL/day, according to manufacturer's label), which was calculated for a 70 kg b.w. adult. The re-estimated dose for rats was 0.28 mL/kg b.w. which was referred to as low dose group. High dose pre-treatment refers to ten times of recommended dose; 2.8 mL/kg b.w. (p.o.).

Electrocardiographic study of Mrityunajay on digoxin-induced arrhythmic rats

The experiment was performed using Edan VET-300 veterinary ECG machine (China). Digoxin arrhythmogenic dose (AD) has been proposed to be 13 ± 1 mg/kg b.w., in adult rats by Weinhouse et al. (1983). This was taken as a reference point to start screening for an arrhythmogenic dose of digoxin for the current studies. Doses of 8, 10, 13, 15, 20 and 22 mg/kg b.w. was administered intraperitoneally in ketamine anesthetized rats and electrocardiogram was monitored continuously for 60 min. Auto (all leads) and rhythm (lead II) was recorded to observe any characteristic arrhythmic changes. A concentration of 20 mg/kg b.w, i.p. was chosen for induction of arrhythmia without causing death for 60 min. Rats were weighed and then anesthetized with ketamine (50 mg/kg b.w., i.p.). After anesthesia, rats were placed on dissecting board filled with wax and pinned to it by small pins. Then electrodes, smeared with electrode gel, were connected to the two forelimbs, two hind limbs, and chest. ECGs recordings were taken for 30 min, after 20 min of ketamine hydrochloride (50 mg/kg b.w., i.p.) injection. The recordings were performed before and 60 min after digoxin (20 mg/kg b.w., i.p.) administration. Arrhythmias were assessed by identifying and quantifying the different changes in heart rate during the 60-min recording period. The ECG was recorded as lead I, II, III, aVR, aVL, aVF and V (chest lead). For this study, only lead II was discussed. A schematic diagram of the experimental process is presented in Figure 1.

Hematological effect of Mrityunajay on digoxin-induced arrhythmic rats

Humalyzer 3000 (Blood analyzer, Germany) was used for this experiment. Rats were fasted overnight before collection of blood. After ECG recording, an incision was made into their thoracic cavities.

Blood samples were collected by aorta puncture using 5 mL hypodermic syringe and dispensed into 1.5 mL microcentrifuge Eppendorf tubes. The samples were allowed to stand for 30 min at room temperature to clot. Serum for the assays was thereafter separated from the clot by centrifugation at 4000 rpm for 5 min using a centrifuge machine.

The supernatant, that is serum was harvested by simple aspiration with Pasteur pipette and transferred into another micro-centrifuge tube. All the biochemical determinations were carried out immediately after separation of the serum from the clot; the serum samples were not stored. Diagnostic kits for the lipid profile (with the exception of low-density lipoprotein-cholesterol, LDL-C) were used according to the manufacturer's instruction for the estimation of serum lipid profile. Serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) were estimated spectro-photometrically using the methods described by Friedewald et al. (1972).

Data analysis

Data were expressed as mean ± S.E.M. (n = 5). Differences in mean values between experimental groups were analyzed by two tailed student's t-test. Differences in mean values between experimental groups were analyzed by one-way ANOVA (analysis of variance) followed by Dunnett's multiple comparison tests where applicable. A probability value less than 0.05 (p<0.05) was defined to be significant.

RESULTS

Body weight observations

Body weight was taken initially on day one and finally on day 36. The result is shown in Table 1. Pre-treatment of Mrityunajay did not alter body weight compared to control group.

Table 1. Effect of drugs on body weight (gm) after pre-treatment of Mrityunajay.

Treatment	Dose (mL/kg)	Initial weight (g)	Final weight (g)	Body weight gain (g)
Control	10	108.75 ± 4.27	130 ± 2.04	21.25 ± 3.15^{ns}
Low dose	0.28	100 ± 4.08	125 ± 3.54	25 ± 3.23^{ns}
High dose	2.8	98.75 ± 3.15	126.25 ± 2.39	27.5 ± 2.5^{ns}

Values are presented as mean ± SEM (n = 5). ns = not significant; *p<0.05 when compared to control group.

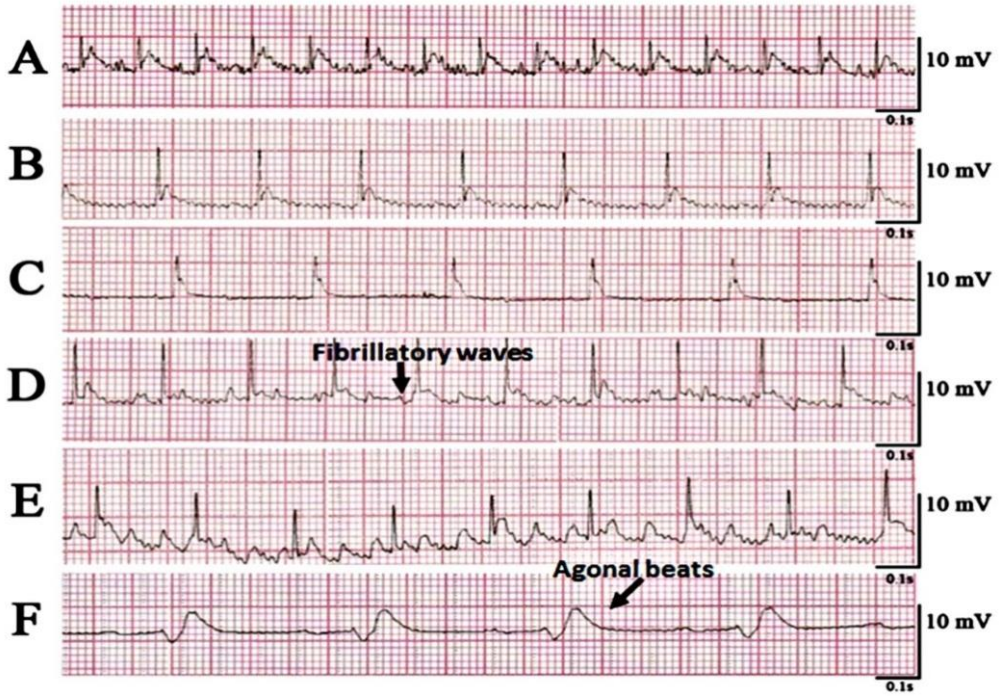

Figure 2. Arrhythmogenic dose (AD) determination by administering varying doses of digoxin (selected tracings: A, 8 mg/kg b.w.; B, 10 mg/kg b.w.; C, 13 mg/kg b.w.; D, 15 mg/kg b.w.; E, 20 mg/kg b.w.; F, 22 mg/kg b.w., i.p.).

Electrocardiographic studies

Determination of arrhythmogenic dose of digoxin

The ECG wave forms were analyzed after intraperitoneal administration of digoxin at the doses ranging from 8-22 mg/kg b.w, i.p. and have been depicted in Figure 2. The dose 8 mg/kg b.w., i.p. did not produce any changes in heartbeat (Panel A); 10-13 mg/kg b.w. exhibited bradycardia (Panel B-C), 15 mg/kg b.w., i.p. induced unstable atrial fibrillation (Panel D), 20 mg/kg b.w., i.p. caused stable induction of arrhythmia, atrial flutter (AF) (Panel E), and 22 mg/kg b.w., i.p. produced agonal beats or dying beats (Panel F) which was an indication of severe digitalis intoxication. Therefore, the dose 20 mg/kg b.w, i.p. of digoxin was selected to induce arrhythmia without causing the death of animals.

Induction of arrhythmia by digoxin

In the whole experiments, the injection of digoxin (20 mg/kg b.w., i.p.) induced atrial fibrillations (AFib) and atrial flutters. It also induced ventricular bigeminy, junctional rhythm (inversed p wave), idioventricular rhythm. Additionally, it showed digitalis effect characterized by ST-sagging. The ECG results are shown in Figure 3.

Effect of low dose of Mrityunjay on ECG tracings

Atrial fibrillation, bigeminy rhythm, and digitalis effect were not seen after pre-treatment of 0.28 mL/kg b.w. of Mrityunjay. Mrityunjay only showed atrial fibrillation briefly, which had a delayed onset compared to the

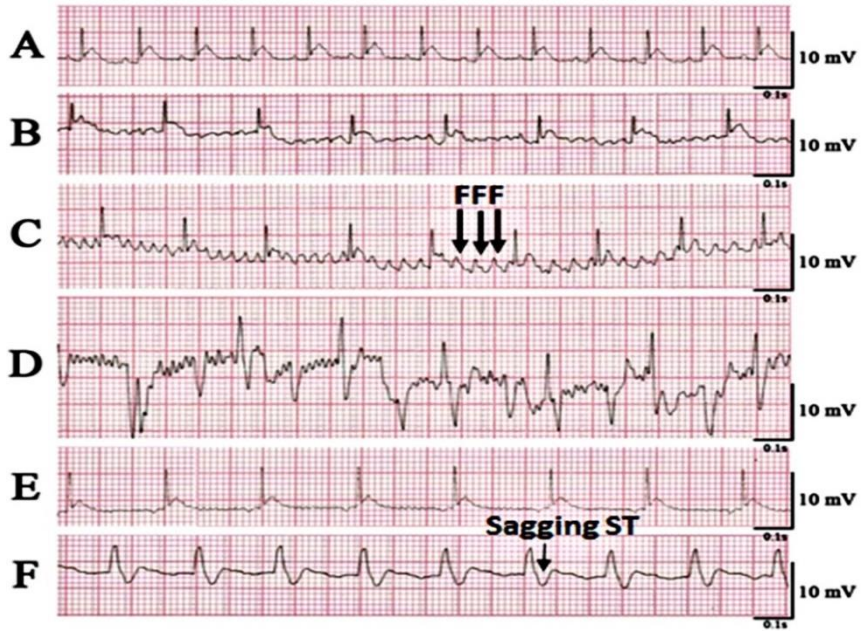

Figure 3. Changes in the electrocardiogram in control group following digoxin administration. Typical ECG tracings of digoxin control group showing NSR (A) and changes after digoxin (20 mg/kg b.w, i.p.) administration (B-F). Panel B showed AFib; (C) AF 5:1 (flutter 'F' waves); (D) bigeminy rhythm; (E) junctional rhythm; (F) digitalis effect, respectively. The recording speed from panel A-F was 50 mm/s. ECG tracings were chosen from one of the five (n = 5) similar and representative experiments. NSR, normal sinus rhythm; AFib = atrial fibrillation; AF = atrial flutter.

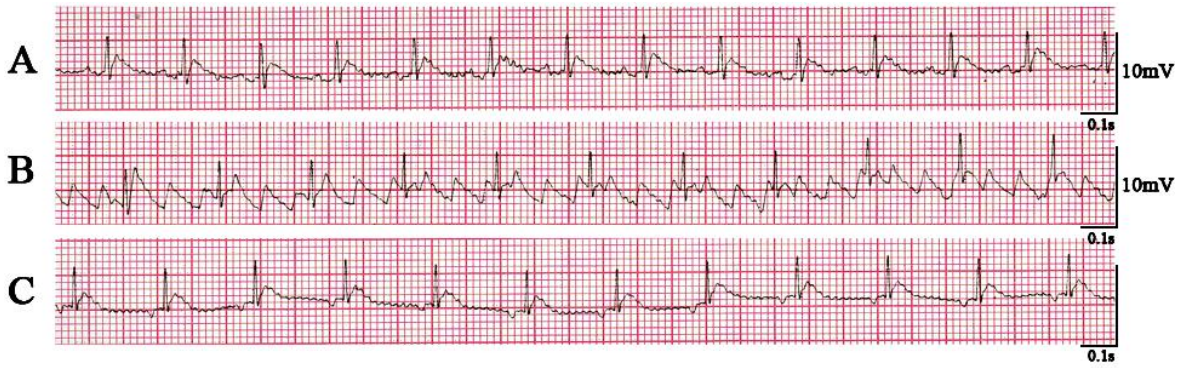

Figure 4. ECG tracings of Mrityunjay pre-treatment (0.28 mL/kg, b.w.) showing NSR (A) changes after digoxin (20 mg/kg b.w., i.p.) administration (B-C). Panel A showed normal sinus rhythm (NSR), panel B showed AFib and panel C showed AF 2:1, respectively. The recording speed from panel A-C was 50 mm/s. ECG tracings were chosen from one of the five (n = 5) similar and representative experiments. NSR, normal sinus rhythm; AFib= atrial fibrillation; AF=atrial flutter.

digoxin-control rats. Atrial fibrillation and other rhythm abnormalities were absent in Mrityunjay pre-treated groups (Figure 4).

Effect of high dose of Mrityunjay on ECG tracings

After pre-treatment of Mrityunjay at the dose of 2.8 mL/kg b.w, p.o., atrial fibrillation, atrial flutter, bigeminy rhythm, and digitalis effect were not observed. Mrityunjay showed no abnormal beats but decreased heart rate following digoxin administration (Figure 5).

Effect of Mrityunjay on heart rate

Heart rate (HR) changes before and after administration of digoxin were noted in digoxin control (group II) and

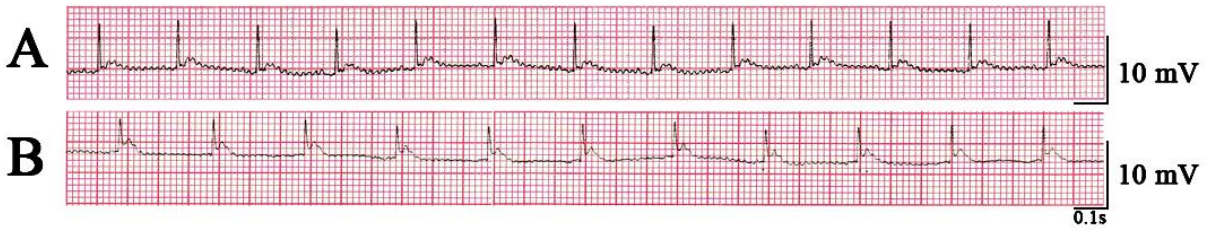

Figure 5. ECG tracings of Mrityunjay (2.8 mL/kg b.w., group IV) showing NSR (A) changes after Digoxin (20 mg/kg b.w., i.p.) administration (B). Panel A showed normal sinus rhythm (NSR), panel B showed sinus bradycardia (SB). The recording speed from panel A, B is 50 mm/s. ECG tracings are chosen from one of the five (n = 5) similar and representative control experiments. NSR, normal sinus rhythm; AFib = atrial fibrillation; AF = atrial flutter.

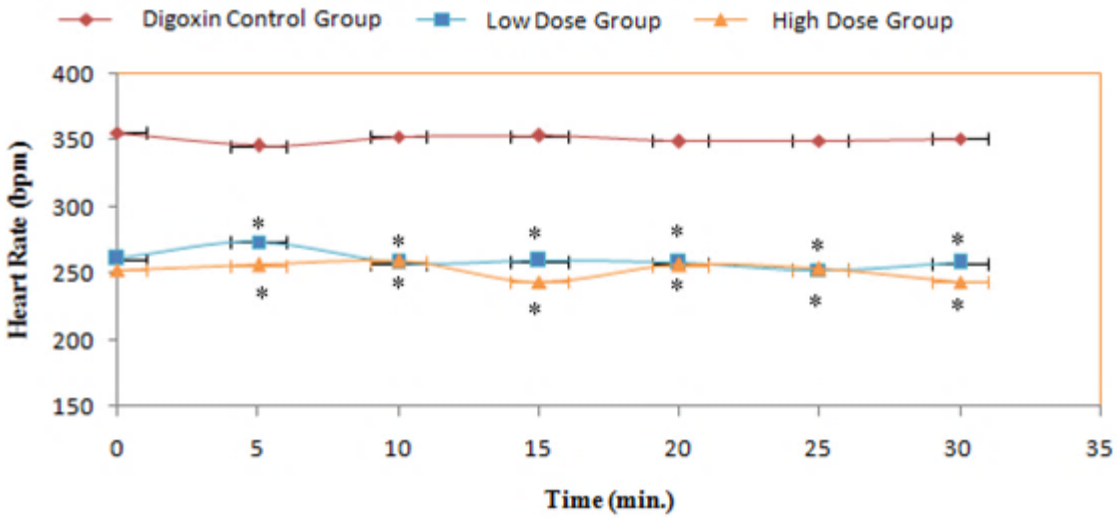

Figure 6. Effect of Mrityunjay treatment (low dose = 0.28 mL/kg b.w, high dose = 2.8 mL/kg b.w.) on heart rate of rodents before digoxin injection (20 mg/kg b.w., i.p.). Values are presented as mean ± SEM (n = 5). *p<0.05, compared to digoxin control group.

chronic Mrityunjay treated (groups III and IV) groups. The result is shown in Figures 6 and 7. Figure 6 shows that Mrityunjay treatment produced significant (p<0.05) stable decrease of HR at both high (2.8 mL/kg b.w., p.o.) and low (0.28 mL/kg b.w., p.o.) dose. Figure 7 shows that Mrityunjay, significantly (p<0.05) prevented reduction of heart rate caused by digoxin as well as stabilized heart rhythm at both oral doses. The effect was dose dependent.

Duration of action of Mrityunjay on ECG

Figure 6 shows the duration of normal sinus rhythm (NSR), sinus bradycardia (SB), atrial fibrillation (AFib), atrial flutter (AF), bigeminy rhythm (BR), junctional rhythm (JR), and digoxin effect (DE) of digoxin which were 8 ± 1.22, 23.5 ± 3.16, 2.25 ± 2.11, 13.5 ± 2.33, 2.75 ± 1.01, 6.5 ± 1.05 and 2.75 ± 1.05 min, respectively. The duration of NSR, SB, AF and JR for Mrityunjay low dose (0.28 mL/kg b.w.) pre-treatment was 7.75 ± 1.03, 43.25 ± 4.11, 3.5 ± 0.22, and 5.5 ± 0.21 min, respectively. Pre-treatment of Mrityunjay with high dose (2.8 mL/kg b.w.) caused 11 ± 2.11, 40.75 ± 4.66, and 8.25 ± 1.18 min of NSR, SB, and JR, whereas, AFib, BR were absent. Mrityunjay pre-treatment induced significantly (p<0.05) SB at both high and low doses compared to digoxin control group. On the other hand, it significantly (p<0.05) reduced AFib, AF and DE at both doses.

Hematological test for serum lipid profile

As depicted in Figure 9, Mrityunjay pre-treatment at a low dose (0.28 mL/kg b.w.) for 35 days did not induce a significant decrease of TC, TG, LDL-C and an increase in the HDL-C level of serum, compared to control group. There was a significant decrease (p<0.05) in serum TC

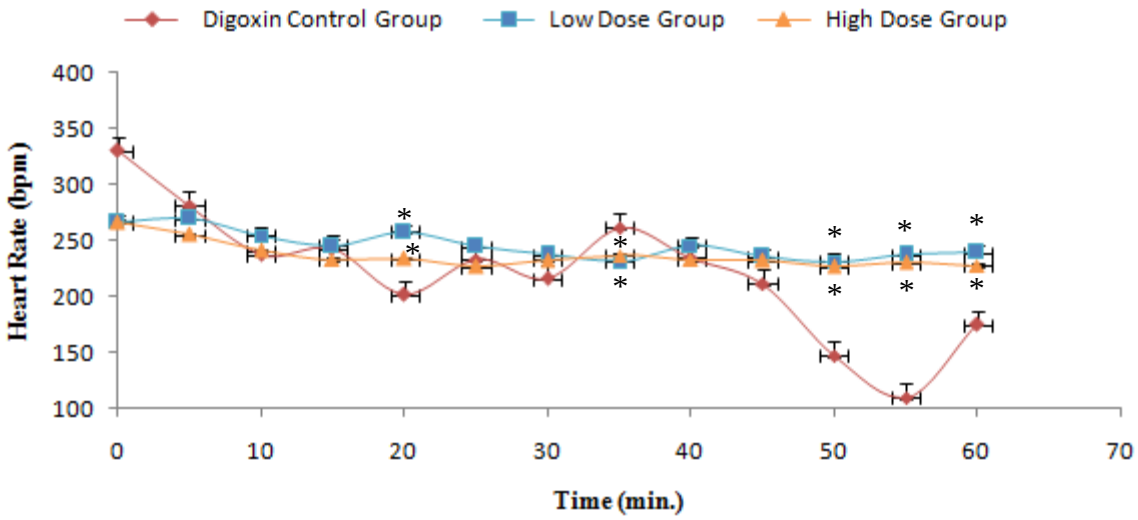

Figure 7. Effect of Mrityunjay (low dose = 0.28 mL/kg b.w.; high dose = 2.8 mL/kg b.w.) treatments following digoxin injection (20 mg/kg b.w., i.p.) on heart rate of rodents. Data are presented as mean ± SEM (n = 5). *p<0.05 when compared to digoxin control group.

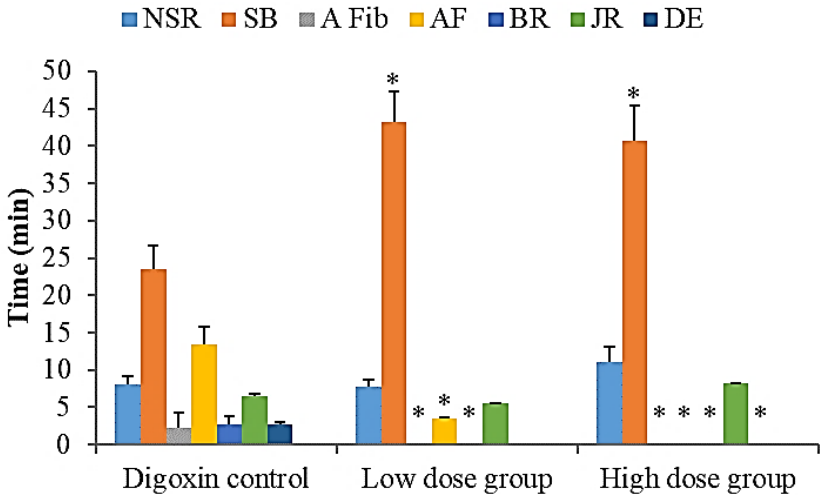

Figure 8. Duration of different changes in ECG in Mrityunjay pre-treated groups (low dose = 0.28 mL/kg b.w., high dose = 2.8 mL/kg b.w.) following digoxin injection (20 mg/kg bodyweight, i.p.). Values are presented as mean ± SEM (n = 5). *p<0.05, compared to digoxin control group. SB = sinus bradycardia; AFib = atrial fibrillation; AF = atrial flutter; BR = bigeminy rhythm; JR = junctional rhythm; DE = digoxin effect.

and LDL-C with the pre-treatment with a higher dose (2.8 mL/kg b.w.) of Mrityunjay. A higher dose of Mrityunjay did not decrease TG significantly but increased the HDL-C level of serum significantly (p<0.05) compared to the control group. The effects of Mrityunjay were dose dependent. In high dose pre-treatment, the decrease in TC, TG, and HDL-C was insignificant where LDL-C was significant (p<0.05) compared to the low dose pre-treatment group.

DISCUSSION

An insignificant increase in body weight was observed between the Mrityunjay pre-treated groups and the control group by five weeks after initiating oral administration (Table 1). It has been reported that digoxin is 100 times more potent in rodents than humans by the means of therapeutic efficacy and toxicity (Gonano et al., 2011). The dose of digoxin (20 mg/kg b.w., i.p.) has been

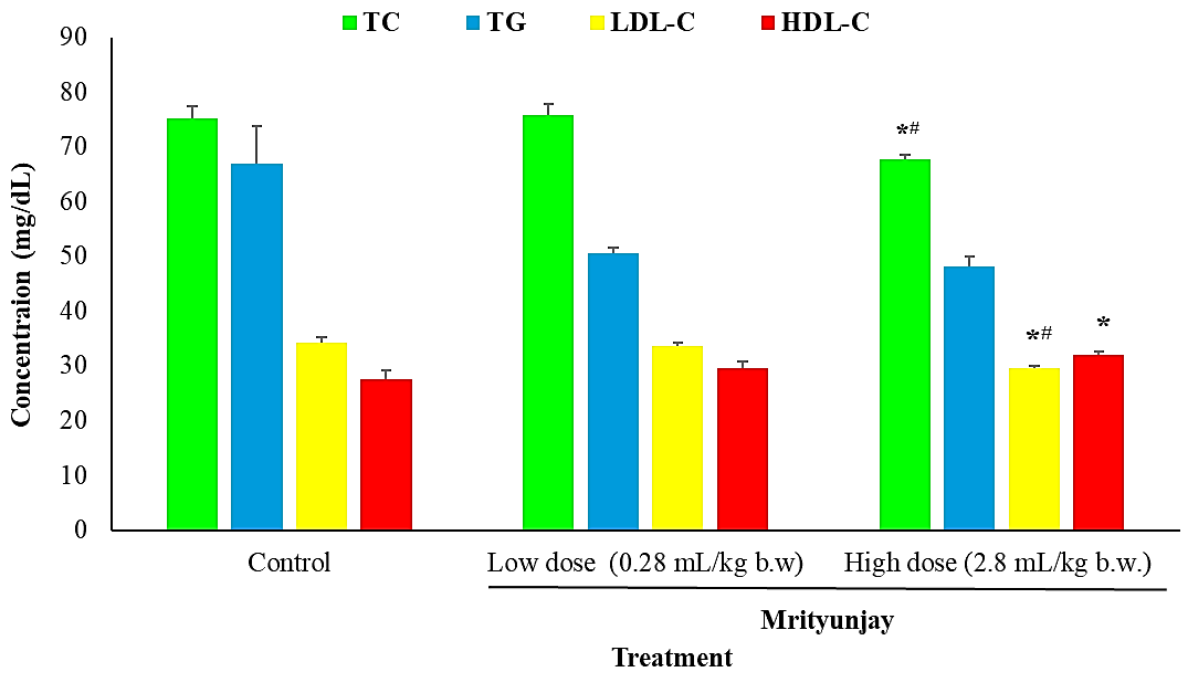

Figure 9. Effect on lipid profile after chronic pre-treatment with Mrityunjay. Data are presented as mean ± SEM (n = 5). $^{*}p<0.05$, compared to control group. $^{\#}p<0.05$, compared to low dose. TC = total cholesterol; TG = triglyceride; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol.

selected by trial and error based approach which systematically and reproducibly produced arrhythmias in experimental rats (Figure 2). In addition, this dose of digoxin has been reported to produce low toxicity in the heart of rats (Dutta and Marks, 1972). In control experiments, it was observed that digoxin at an order of magnitude below the dose used, did not promote arrhythmias within the first hour following administration, whereas digoxin at an order of magnitude above the one used, killed the animals during the first few minutes.

Atrial fibrillation, bigeminy rhythm, and digitalis effect were absent in Mrityunjay low dose pre-treated group (Figure 4). Mrityunjay only showed atrial fibrillation briefly, which had a delayed onset compared to the digoxin-control rats. Atrial fibrillation and other rhythm abnormalities were absent in Mrityunjay pre-treated groups. High dose pre-treatment of Mrityunjay did not produce atrial fibrillation, atrial flutter, bigeminy rhythm, and digitalis effect was absent in the group (Figure 5). The pre-treatment showed a decrease in heart rate but no abnormal beats before and after digoxin administration (Figures 6 to 7). Therefore, it can be suggested that Mrityunjay successfully inhibited digoxin-induced arrhythmia at both doses.

Oral pre-treatment of Mrityunjay significantly ($p<0.05$) as well as completely inhibited atrial fibrillation and bigeminy rhythm (Figure 8) at both high and low dose level. It significantly ($p<0.05$) delayed onset as well as decreased the duration of atrial flutters. Furthermore, Mrityunjay inhibited reduction of heart rate which was induced by digoxin and stabilized heart rhythm (Figures 7 to 8). The effect could be attributed to the negative chronotropic action of Mrityunjay. However, Mrityunjay showed marked bradycardia following induction of arrhythmia. This is possibly due to the presence of *rauwolfia* alkaloids in the preparation, which shows bradycardia, particularly when used concurrently with digitalis (Barnhart, 1991). Digoxin inhibits Na^{+}/K^{+}-ATPase for a prolonged period of time that in turn activates the Na^{+}/Ca^{2+} exchanger which increases the intracellular concentration of the Ca^{2+} ion. Ajmaline, an anti-arrhythmic drug, present in *R. serpentina* may contribute to this effect (Kiesecker et al., 2004).

Lower dose pre-treatment of Mrityunjay did not exhibit significant changes in serum lipid profile of rats (Figure 9). However, chronic pre-treatment of Mrityunjay at a higher dose (2.8 mL/kg b.w., p.o.), significantly ($p<0.05$) decreased TC and increased in HDL-C level of rat serum. The decrease in TC may be attributed to the presence of *rauwolfia* alkaloids and possible inhibition of cholesterol biosynthesis by down regulation of HMG-CoA reductase, inhibiting mevalonic acid pathway (Rand and Jurevics, 1977; Lüllmann, 2005). The present study also determined LDL-C/HDL-C and TC/HDL-C ratio of blood serum after Mrityunjay pre-treatment (Table 2). Increased value of these indexes indicates progression to the lower risk of coronary artery diseases (Levy et al., 1984). Oral administration of Mrityunjay increased the ratio between

Table 2. Serum lipid parameters of Mrityunjay.

Treatment	Dose (mL/kg b.w.)	HDL-C/LDL-C	HDL-C/TC
Control	10	0.81 ± 0.05	0.37± 0.01
Low dose	0.28	0.88 ± 0.02	0.39 ± 0.01
High dose	2.8	0.93 ± 0.01	0.47 ± 0.01*

Data are presented as mean ± SEM (n = 5). *p<0.05, compared to control group.TC = total cholesterol; TG = triglyceride; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol.

HDL-C and TC as well as LDL-C in both doses. The results indicate that Mrityunjay could be effective in lowering the risk of coronary artery diseases.

Conclusions

Cardiovascular diseases are now a major health risk in developing countries, like Bangladesh. Ayurvedic formulations are frequently used in the management of cardiac diseases. Although demand for ayurvedic medicines is increasing gradually, their safety and efficacy are still a major concern. While the active constituents of the majority of these plants are well investigated, the specific active constituents for a particular therapeutic efficacy in the plant-based herbal medicines, formulated with several plant species, are still ill established. The present study revealed that ayurvedic preparation, Mrityunjay has a definite and dose dependent modulatory effect on the heart. It may exert potent cardio-protection when subjected to digoxin-induced arrhythmia. It could also lower triglyceride levels significantly. Therefore, considering the potential bioactivity, this drug can be further screened to rationalize its medicinal use.

Ethical approval

All experiments were performed according to the ethical standards laid down in the Declaration of Helsinki 2013. Animals were handled and treated according to the principles of the Swiss Academy of Medical Sciences and Swiss Academy of Sciences. Animals were euthanized according to the Guidelines for the Euthanasia of Animals: 2013 edition.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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