The Journal of Basic & Applied Zoology (2016) **76**, 1–6

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The Journal of Basic & Applied Zoology

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In vivo pretreatment of *Eudrilus eugeniae* powder attenuates β-adrenoceptor toxicity mediated by isoproterenol in rat model

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Received 12 November 2015; revised 17 February 2016; accepted 25 February 2016

KEYWORDS

Lipids;
Myocardial infarction;
Isoenzymes;
Eudrilus eugeniae;
Cholesterol;
Isoproterenol

Abstract The present study was designed to discover the potential cardioprotective function of earthworm powder (EWP) extracted from *Eudrilus eugeniae* on isoproterenol (ISO)-induced myocardial infarction in male Wistar rats. The rats were divided into four groups, with six rats in each group. Certain rats were pretreated with EWP (200 mg/kg bwt) (Group III), and a myocardial infarction was then induced by subcutaneous injection of ISO (85 mg/kg bwt) (Group II). Oral pretreatment of 200 mg/kg bwt of EWP for 28 days significantly ($p > 0.05$) improved the blood profile levels, including (a) the lipid profile of total cholesterol (TC), free fatty acids (FFA), and triglycerides (TG); (b) low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), high-density lipoprotein (HDL), and protein; and (c) A/G ratio, glucose and uric acid levels. The electrophoretic pattern of elevated lactose dehydrogenase (LDH) levels was recovered by EWP treatment as evidenced by comparison with ISO-induced rats with cardiac damage. The above results indicate that EWP (200 mg/kg bwt) provides a cardioprotective effect by attenuating the blood profile, lipid profile, biochemical levels, and LDH patterns in rats that experienced an ISO-induced myocardial infarction.

Introduction

Myocardial infarction (MI) is a condition characterized by an imbalance between the myocardial oxygen supply and demand (Mohanty et al., 2004). Accumulating evidence suggests that reactive oxygen-derived free radicals play a crucial role in the

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Peer review under responsibility of The Egyptian German Society for Zoology.

http://dx.doi.org/10.1016/j.jobaz.2016.02.003

pathogenesis of isoproterenol (ISO)-induced myocardial infarction (Nirmala and Puvanakrishnan, 1996). The effects of ISO on the myocardium are mediated through β1 and β2 adrenoceptors. Both β1 and β2 adrenoceptors mediate the positive isotropic and chronotropic effects of β adrenoceptor agonists. Thus, ISO produce relative ischemia or hypoxia due to myocardial hyperactivity and coronary hypotension (Brodde, 1991). ISO additionally causes myocardial ischemia due to the excessive production of free radicals resulting from the oxidative metabolism of catecholamine (Gunjal et al., 2010). Further toxic dosages of ISO cause characteristic myocardial damage that subsequently results in heart failure (Liu et al., 2012).

Myocardial proteins, lipids, and DNA play an important role in cardiovascular diseases by modifying the composition, structure, and stability of cell membranes. Studies have shown that high levels of total cholesterol, triglycerides, and low-density lipoprotein cholesterol and low levels of high-density lipoprotein cholesterol are risk factors for cardiovascular diseases. Reactive oxygen species may contribute to the events associated with atherogenesis and lead to the progression of atherogenic lesions by promoting the oxidation of low-density lipoproteins (Upaganlawar and Balaraman, 2012). Lactate dehydrogenase (LDH) is an enzyme present in a wide variety of organisms, including plants and animals that exist as five isoenzymes (LD1, LD2, LD3, LD4, and LD5). The differential distribution of LDH isoenzymes enables its use in differential diagnosis, as a marked increase in the proportion of LD1 in the serum indicates myocardial infarction (Nigam, 2007).

A better understanding of the processes involved in myocardial infarction has stimulated the search for new drugs that could limit myocardial injury. Recently, earthworms have been the subject of widespread investigation because they have unique therapeutic properties, including anti-inflammatory, anti-oxidative, antitumor, and anti-bacterial effects (Balamurugan et al., 2007). Earthworm powder (EWP) of *Eudrilus eugeniae* is a good source of protein, carbohydrates, fat, micro and macro minerals, vitamins, and antioxidants (Anitha and Jayraaj, 2012). The free radical scavenging potential of EWP helps to reduce the oxidative damage induced by ISO and to restore both enzymatic and non-enzymatic antioxidants in rat models without any adverse side effects (Anitha and Jayraaj, 2014). Based on these facts, in this study, we investigated the cardioprotective activity of EWP obtained from *E. eugeniae* against ISO-induced myocardial infarction in rats.

Methods and methods

Materials

The living earthworm *E. eugeniae* was collected from Aarthi farms, Kondegoundan palayam village, Pollachi Taluk, Coimbatore District, Tamil Nadu, India. Isoproterenol hydrochloride, filter papers, solvents and all other chemicals were of analytical grade and were acquired from Sigma–Aldrich Chemicals, Mumbai, India. Double distilled water was used in all the biochemical assays.

Extraction and preparation of earthworm powder

The earthworms were washed with running tap water to remove any dirt from the body surface. The earthworms were kept in 0.5% NaCl at room temperature for 1–2 h with a few solution changes until their digestive systems were clean. The cleaned earthworms were taken out of the solution and minced with scissors. Fifteen grams of earthworm tissue was homogenized in 40 ml of chloroform–methanol (v/v) solution and left overnight at 4 °C. The following day, 16 ml of distilled water was added to the homogenate. The solution was mixed and centrifuged at 2460*g* for 10 min. Three clearly visible layers were obtained. The upper water/methanol layer was removed by pipette and evaporated on a rotary evaporator until the methanol was vaporized, and an opalescent solution was subsequently obtained at pH7. The solution was then freeze-dried, and the earthworm powder (EWP) was stored at 4 °C (Hrzenjak et al., 1998).

Experimental animals

Purebred healthy male Wistar albino rats weighing 150–180 g were procured from the Small Animal Breeding Centre, Agricultural University, Mannuthy, Kerala. Animals were acclimatized under standard laboratory conditions at 25 ± 2 °C and a normal photoperiod (12 h light:dark cycle). The animals had *ad libitum* access to standard rat chow and water. The food was withdrawn 18–24 h before the experiment. The care and use of laboratory animals were conducted according to the guidelines of the Council Directive CPCSEA, India (No: 659/02/a), on Good Laboratory Practice (GLP) for animal experimentation. All the animal experiments were performed in the laboratory according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC).

Experimental design

The experimental animals were divided into the following four groups, each containing six Wistar albino rats, which were analyzed for a total experimental period of 32 days. Group I served as a control. In Group II, rats were treated with isoproterenol (85 mg/kg bwt administered subcutaneously twice at an interval of 24 h) dissolved in normal saline. In Group III, rats were orally pretreated with EWP (200 mg/kg bwt) dissolved in saline for a period of 30 days and then treated with isoproterenol (85 mg/kg bwt administered subcutaneously twice at an interval of 24 h) at the end of the treatment period on the 31st and 32nd days. In Group IV, rats were pretreated with EWP (200 mg/kg bwt) dissolved in saline for a period of 30 days. After the experimental regimen, the rats were sacrificed by cervical decapitation under mild chloroform anesthesia. Blood was collected into clean centrifuge tubes by carotid bleeding and allowed to clot. The serum was then separated by centrifugation at 3000 rpm for 15 min and stored at 4 °C for various biochemical and hematological assays.

Hematological and biochemical assays

Hemoglobin (Hb) (Drabkin and Austin, 1932), red blood cells (RBC), white blood cells (WBC) (Chesbrough and McArthur, 1972), platelets (Auto analyzer model Cell-Dyn-1700, No. 41884V96, CD-1700-CS, GMI Incorporation, Ramsey, MN, USA), protein, albumin, globulin, and uric acid were evaluated using standard protocols described by Lowry et al. (1951), Wolfson (1948), Caraway (1963).

Lipid profile estimation in serum

Ten milliliters of a chloroform–methanol (2:1 v/v) mixture was added into a known volume of serum and mixed well for 30 min. The mixture was filtered through Whatman filter paper (No. 42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline, and the mixture was kept overnight in undisturbed conditions. The lower phase containing the lipids was drained off into pre-weighed beakers, and the upper phase was re-extracted with additional chloroform–methanol mixture. The extracts were pooled and evaporated under vacuum at room temperature. The lipid extract was re-dissolved in 3 ml of a chloroform–methanol (2:1) mixture, and aliquots were removed to estimate the serum lipid concentrations. Total cholesterol (Parekh and Jung, 1970), triglycerides (Rice, 1970), free fatty acids (Horn and Menahan, 1981), phospholipids (Rouser et al., 1970) and HDL (Warnick et al., 1985) were assayed in each serum sample, and the lipids were extracted via the method reported by Folch and Lees (1957).

Separation of LDH isoenzymes in serum by electrophoresis

LDH isoenzymes were analyzed based on the *in vivo* toxicity mediated by ISO with the method of gel electrophoresis where 1% agarose gels were prepared and the experimental and control serum samples were loaded into successive wells. After electrophoresis, the gel was removed and stained by the following method: the gel was incubated with staining solution at 37 °C in the dark for a suitable period; the staining solution contained 1.0 ml of 1.0 M lithium lactate, 1.0 ml of 0.1 M sodium chloride, 1.0 ml of 5.0 mM magnesium chloride, 2.5 ml of 0.1% (w/v) nitro blue tetrazolium (NBT), 0.25 ml of 0.1% phenazine methosulfate, 2.5 ml of 0.5 M phosphate buffer, pH 7.5, and 10 mg of NAD in a total volume of 10 ml. The separated LDH isoenzymes appeared as purple bands. Finally, the gel was washed with 7.5% acetic acid, preserved in 5% acetic acid, and scanned using a densitometer (Mckenzie and Henderson, 1983).

Data analysis

The results are expressed as the mean ± SEM. One-way ANOVA was performed, and the statistical comparisons among the groups were performed with Turkey's test using statistical analysis software (SPSS 10.0 for Windows). Differences among the means were analyzed by Duncan's multiple range test (DMRT) at the 5% level ($p < 0.05$).

Result

Valuation of hematological and biochemical parameters

Table 1 shows the results of a hematological assay of the blood from the control and ISO-mediated rats that were treated or not with EWP from *E. eugeniae*. RBC levels were decreased in rats in Group II compared to control (Group I) rats. A simultaneous increase in hemoglobin content and hematocrit value was noticed in Group I animals, the condition was normalized by the treatment of EWP of *E. eugeniae* at 200 mg/kg bwt. Furthermore, as shown in Fig. 1, the biochemical parameters in ISO-mediated rats showed that the level of total protein and the A/G ratio in the serum decreased significantly and the glucose and uric acid contents in the serum increased compared with the control rats ($p < 0.05$).

Lipid profile assessment

Table 2 depicts the lipid profiles of the sera of the control and ISO-treated rats that were treated or not with EWP from *E. eugeniae*. The ISO-treated rats showed significantly increased levels of TC, FFA, TG, PL LDL and VLDL, which were associated with a significant decrease in HDL levels compared to the control rats ($p > 0.05$). The ISO-treated rats in Group III that were orally pretreated with EWP (200 mg/kg bwt) showed a significant ($p < 0.05$) decrease in the levels of TC, FFA, TG, PL, LDL, and VLDL that was associated with a significant increase in HDL compared to ISO-treated rats with cardio toxicity; these levels, except for the HDL and LDL levels, were close to those in the control rats.

LDH isoenzyme expression

ISO (85 mg/kg bwt) treatment caused increased expression of LDH isoenzymes, predominantly LDH1, compared to the control rats (Fig. 2). The elevation of these LDH isoenzymes was normalized with the EWP treated Group III animals.

Discussion

Blood is a good indicator of the health of an organism. It also acts as a pathological indicator of the whole body and hence

Table 1 ISO-mediated changes in the levels of the hematological parameters in control and experimental rats and their attenuation by earthworm powder (*E. eugeniae*).

Groups	Hb (g%)	Platelets (k/μl)	WBC ($\times 10^6$/μl)	RBC ($\times 10^9$/μl)
Group I	13.57 ± 0.32	3.51 ± 0.23	2.55 ± 0.26	7.56 ± 0.21
Group II	17.48 ± 0.26[a,*]	5.61 ± 0.27[a,*]	3.75 ± 0.16[a,*]	6.40 ± 0.24[a,*]
Group III	14.56 ± 0.23[b]	3.46 ± 0.35[b]	2.55 ± 0.27[b]	7.41 ± 0.25[b]
Group IV	14.45 ± 0.34[c]	3.49 ± 0.31[c]	2.70 ± 0.18[c]	7.46 ± 0.32[c]

Values are expressed as the mean ± SD of the six animals in each group. Comparisons with [a]Group II and [b]Group III. [c]Group IV and Group I. *Groups:* Group I: Control; Group II: ISO (85 mg/kg bwt); Group III: ISO (85 mg/kg bwt) + EWP (200 mg/kg bwt); and Group IV: EWP alone (200 mg/kg bwt).

[*] Statistically significant at $p < 0.05$.

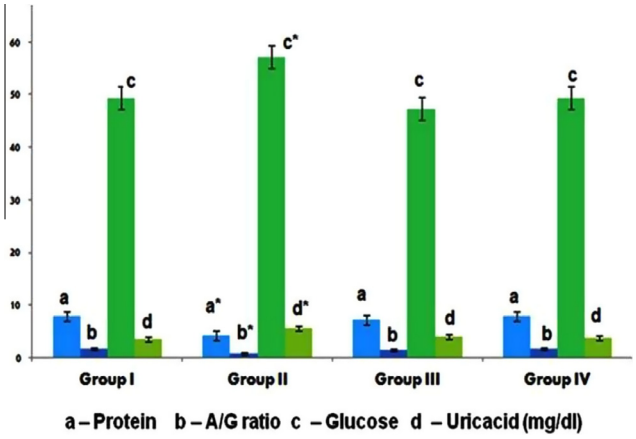

Figure 1 Evaluation of serum protein, A/G ratio, glucose and uric acid levels in ISO-treated and control rats. Group I: control; Group II: ISO (85 mg/kg bwt); Group III: ISO (85 mg/kg bwt) + EWP (200 mg/kg bwt); and Group IV: EWP alone (200 mg/kg bwt). Each column represents the mean ± SD of the six animals in each group. *Statistically significant at $p < 0.05$.

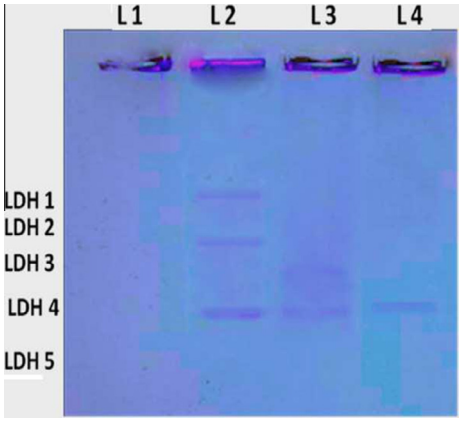

Figure 2 Electrophoretic pattern of elevated LDH isoenzyme levels by the free radical damage induced by ISO, normalization of isoenzymes by EWP treated at 200 mg/kg bwt and control rats. L1, Group I rats (control); L2, Group II rats treated with ISO; L3, Group III rats orally pretreated with 200 mg/kg bwt of EWP and treated with ISO; L4, Group IV rats treated with 200 mg/kg bwt of EWP.

hematological parameters are important for diagnosing the functional status of an animal exposed to toxicant (Joshi et al., 2002). Anemia treatment may be an important part of MI taking this in account, the results of hematological assay of the blood from the control and ISO-mediated rats showed decreased levels of RBC in Group II rats compared to control rats, in accordance with the previous report by Cemin et al. (2011). A significant increase in the hemoglobin content and hematocrit value was found in ISO-mediated rats following treatment with the EWP of *E. eugeniae* compared with the control, which is in accordance with the study by Tarasov (1976), who showed a significant increase in total blood parameters in acute myocardial infarction (AMI) patients compared to normal subjects.

A decrease in serum proteins is usually due to the decrease in albumin or sometimes gamma globulin that occurs due to infections, burns, stress, or heart attacks (Gowenlock et al., 2002). The ISO-induced myocardial infarctions in the rats in Group II may have generated free radical mediated damage that may lead to the production of more oxygen and hydrogen peroxide ions, which in turn could bind to albumin and destroy serum proteins (Nivethetha et al., 2009). ISO-induced free radical generation, which tends to reduce the protein levels and A/G ratio and increase glucose levels in rat serum, was reversed by treatment with EWP at 200 mg/kg bwt. Therefore, MI might be due to an increased release of free radicals from the damaged myocardium into the systemic circulation. This is in agreement with the findings of Upaganlawar and Balaraman (2012), who suggested that lycopene increases protein levels and the A/G ratio and decreases glucose levels in ISO-treated rats.

Tissue is disturbed under hypoxic conditions, and the enzyme xanthine dehydrogenase is converted into xanthine oxidase by the oxidation of the essential SH group. Xanthine oxidase catalyzes the conversion of hypoxanthine to xanthine, uric acid, and superoxide (Rajadurai and Prince, 2007). This might be the reason for the elevation in the uric acid level in the present study. Rats treated with EWP (200 mg/kg bwt) (Group III) can undergo xanthine metabolism to alleviate the toxicity induced by isoproterenol; this relief from toxicity is similar to the normalization of serum uric acid levels in

Table 2 ISO-mediated changes in the levels of cholesterol, triglycerides, free fatty acids, phospholipids, HDL, LDL and VLDL in the serum of the Wistar rat model and their attenuation by earthworm powder (*E. eugeniae*).

Parameters (mg/dl)	Control	ISO	EWP + ISO	EWP alone
TC	89.63 ± 0.02	125.35 ± 1.4[a]	86.33 ± 0.20[b]	89.61 ± 0.02[c]
FFA	42.15 ± 0.03	73.23 ± 0.16[a,*]	41.3 ± 0.01[b]	42.05 ± 0.03[c]
TG	25.83 ± 18.06	66.3 ± 0.02[a,*]	24.31 ± 0.02[b]	25.43 ± 18.06[c]
PL	99.25 ± 0.01	116.28 ± 0.02[a,*]	98.36 ± 0.04[b]	99.25 ± 0.01[c]
HDL	46.03 ± 0.62	31.29 ± 0.99[a,*]	58.96 ± 1.48[b]	46.03 ± 0.62[c]
LDL	54.16 ± 1.03	96.16 ± 1.15[a,*]	60.10 ± 1.39[b]	54.16 ± 1.03[c]
VLDL	11.15 ± 1.22	15.25 ± 0.22[a,*]	10.95 ± 1.75[b]	11.15 ± 1.22[c]

Values are expressed as the mean ± SD of the six animals in each group. Comparisons with [a]Group II and [b]Group III. [c]Group IV and Group I. *Groups:* Group I: Control; Group II: ISO (85 mg/kg bwt); Group III: ISO (85 mg/kg bwt) + EWP (200 mg/kg bwt); and Group IV: EWP alone (200 mg/kg bwt).

[*] Statistically significant at $p < 0.05$.

ISO-treated rats after pretreatment with naringin (Abirami and Kanagavalli, 2013).

High levels of circulating cholesterol and its accumulation in heart tissue are strongly associated with cardiovascular damage (Deepa and Varalakshmi, 2005). In the present investigation, ISO-treated rats (Group II) showed an increase in the levels of TC, FFA, TG, PL, LDL, and VLDL that was associated with a significant decrease in serum HDL compared to control rats. ISO administration has been reported to stimulate adenylate cyclase activity, resulting in enhanced cAMP formation, which increases lipid biosynthesis and leads to hyperlipidemia via phospholipase A2 in circulation (Franson et al., 1972).

The higher levels of serum free fatty acids in animals treated with ISO are reported to result from the increased levels of lipolysis (Patil et al., 2007). LDL carries most of the cholesterol in the plasma, and increases in LDL levels depend on increasing levels of total cholesterol. Several studies have indicated that the ratios of LDL-cholesterol/HDL-cholesterol and total cholesterol/HDL-cholesterol can be good indexes of atherosclerotic injury in the walls of vessels (Ithayarasi and Devi, 1997). The high levels of VLDL in ISO-treated animals could be due to the decreased activity of extra hepatic lipoprotein lipase (Prabhu et al., 2006). The contents of TC, FFA, TG, PL, LDL, VLDL, and HDL were brought to near-normal levels in EWP-treated rats (200 mg/kg bwt) potentially due to the membrane-stabilizing activity of EWP. This effect may increase the capacity of myocytes to regenerate new phospholipids, which are necessary to repair damaged membranes.

The measurement of serum LDH isoenzymes by agarose gel electrophoresis is necessary for greater specificity in assessing cardiac injury because a non-specific increase in total LDH isoenzymes in serum causes tissue damage. Due to the necrosis induced by ISO, the heart-specific isoenzyme LDH1 could be released into the blood circulation. In the present study, an increase in the LD1, LD2, LD3, LD4, and LD5 isoenzyme bands in rats with an ISO-mediated myocardial infarction was observed, which is supported by the previous findings of Prabhu et al. (2006), who stated that mangiferin exerts a beneficial effect on the LDH pattern in rats with an ISO-induced MI. EWP is rich in phenol, which possesses antioxidant activity (Anitha and Jayraaj, 2012), and this could contribute to its mechanism of action to protect the myocardium from damage and to prevent the leakage of lipids and LDH isoenzymes into the blood. These results are supported by the results of the rats in Group IV, who were pretreated with 200 mg/kg bwt of EWP alone.

Conclusion

Finally the EWP treatment of ISO-mediated rats (200 mg/kg bwt) had a tendency to ameliorate the changes mediated by ISO without any adverse side effects and hence, these results help us to understand the pharmacological potential of EWP in normalizing the parameters analyzed in rat model. The isolation of the pure compound responsible for these stabilizing properties is now in progress.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgements

The authors thank the University Grants Commission (F.No. 38/111 dated 19.12.2009), New Delhi, India, for the financial assistance as a project fellow to Dr. J. Anitha. The authors thank the Deanship of Scientific Research at King Saud University for its funding through project No. RGP-1435-057. We also thank the Research and Development Centre (R&D), Bharathiar University, Coimbatore, for providing instrumentation facilities.

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