Research Article

Protective Effect of Earthworm Powder *(Eudrilus Eugeniae)* on Cardiac Markers and Histopathological Views of Isoproterenol Induced Wistar Male Rats

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ABSTRACT
The study was intended to appraise the shielding effect of earthworm powder (EWP) against isoproterenol induced myocardial infarction in wistar male rats. The animals were orally pretreated with EWP (200mg/kg b.wt.) (group III) for 30 days and then intoxicated with isoproterenol (85 mg/kg b.wt. administered subcutaneously twice at an interval of 24 hrs) (group II). Group IV animals were pre-treated with atrovastatin (40mg/kg b.wt. administered subcutaneously twice at an interval of 24h) for 30 days, 85 mg/kg b.wt. of isoproterenol was given by subcutaneously injection twice at an interval of 24h. Animals were orally pretreated with 200mg/kg b.wt EWP for 30 days (group V). Isoproterenol intoxicated myocardial infarction was confirmed by disturbances in serum and cardiac markers namely creatinine kinase (CK), creatinine kinase – muscle brain (CK-MB), and troponin T (CnTn), aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). ISO induced rats showed specific amelioration in the levels of these enzymes in serum and heart which tends them to get elevated in myocardium. The levels of these enzymes accomplish normalcy as observed in EWP pretreated rats. Histopathological evidence also supports the curing effect of EWP against isoproterenol. Pretreatment with EWP ameliorate the elevation of ISO induced pathological changes, reduced the lipid peroxide formation and retained the myocardial maker enzyme activities at near normal. Thus the result at hand is in concordance with the view that EWP is proficient in combating myocardial free radical damage aggravated by ISO thus proving its protective role.

Keywords: Cardiac markers, earthworm powder, *Eudrilus eugeniae*, myocardial infarction, isoproterenol

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INTRODUCTION

Globally, myocardial infarction (MI) is a major public health concern and the leading cause of mortality [1]. It is one of the leading causes of death for both men and women worldwide [2]. Due to changing lifestyles in developing countries, such as India, and particularly in urban areas, MI is making an increasingly important contribution to mortality statistics [3]. MI is an acute or chronic form of cardiac disability arising due to the imbalance between the myocardial supply and demand for oxygenated blood results in irreversible necrosis of heart tissue that is responsible for the principle cause of death in developed and developing countries [4].

Isoproterenol (ISO) induced myocardial necrosis is a well known standard model to study the beneficial effects on cardiac dysfunctions, as the pathophysiological changes following isoproterenol administration are comparable to those taking place in human MI [5]. ISO is a ß-adrenergic agonist that causes severe stress in myocardium and necrotic lesions in the heart muscles. ISO induced myocardial injury involves membrane permeability alterations, which brings about the loss of functions and integrity of myocardial membranes [6].

By studying the biochemical alterations and histopathological changes that takes place in an animal model, it is possible to gain more knowledge in to the mechanisms leading to the attested metabolic process in human myocardial infarction. Myocardial cell protection and prevention of cell ischemia/necrosis have been therapeutic targets for a long time. Although modern drugs are effective in preventing cardiovascular disorders, their use is often limited because of their side effects [7]. Hence, the entire world population is turning towards natural drugs because of the widespread belief that "natural medicines" are healthier and safer than synthetic ones [8].

Earthworms have been widely used in traditional Chinese medicine for thousands of years [9]. Earthworm protein and its coelomic fluid were reported to exhibit cytolytic, proteolytic, haemolytic, mitogenic, tumorstatic, antibacterial and anti-inflammatory activities. Earthworm has potent enzymes, which have strong fibrinolytic and thrombolytic activities [10]. Hence in the present study, we induced MI by isoproterenol in wistar male rats and studied the protective effects of earthworm powder (EWP).

Materials and Methods

Collection of earthworm

The earthworm namely *Eudrilus eugeniae* was collected from Aarthi farms, Kondegoundanpalayam village, Pollachi Taluk, Coimbatore District, Tamil Nadu, India. The collected species were cultured under optimal conditions at Kongunadu Arts and Science College Premises, Coimbatore - 641 029, Tamil Nadu, India, for further use.

Extraction and Preparation of the sample

The earthworms were washed with running tap water to remove any dirt from body surface. The earthworms were kept in 0.5% NaCl at room temperature for 1-2h with few changes of solution until their digestive systems were clean. Animals were taken out of the solution and minced with scissors. Three grams of earthworm tissue were homogenized in 40ml of chloroform-methanol (v/v) solution and left overnight at 4°C. The following day, 16ml of distilled water was added to the homogenate. It was mixed and centrifuged at 2460Xg for 10 min. Three clearly visible layers were obtained. The upper, water/methanol layer was taken out by pipette and evaporated on a rotary evaporator until methanol was left. An opalescent fluid, pH 7, was obtained. It was freeze dried and the earthworm powder (EWP) was kept at 4°C until use [11].

Experimental Animals

Male wistar albino rats, weighing 150–180 g, procured from the Small Animal Breeding Centre, Agricultural University, Mannuthy, Kerala were used. Animals were acclimatized under standard laboratory conditions at 25° ± 2 °C and normal photoperiod (12 h light: dark cycle). The animals were fed with standard rat chow and water ad libitum. The food was withdrawn 18–24 h before the experiment.

The care and use of laboratory animals were done according to the guidelines of the Council Directive CPCSEA, India (No: 659/02/a) about Good Laboratory Practice (GLP) on animal experimentation. All animal experiments were performed in the laboratory according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC).

Sources of chemicals

All chemicals were of analytical grade and chemicals required for sensitive biochemical assays were obtained from Sigma–Aldrich chemicals, Mumbai, India. Double distilled water was used in all biochemical assays.

Study design

The experimental animals were divided into four groups, each group containing six animals, analyzed for a total experimental period of 32 days as follows:

Group 1: Normal control rats fed with standard rat chow and pure drinking water;

Group 2: Rats were administered with isoproterenol (85 mg/kg b.wt. administered subcutaneously twice at an interval of 24h) dissolved in normal saline.

Group 3: Rats were pretreated with EWP (200mg/kg b.wt.) dissolved in saline for a period of 30 days and then administered with isoproterenol (85 mg/kg b.wt. administered subcutaneously twice at an interval of 24h)

Group 4: Rats were pre-treated with atrovastatin (40mg/kg b.wt. administered subcutaneously twice at an interval of 24h) for 30 days, 85 mg/kg b.wt. of isoproterenol was given by subcutaneously injection twice at an interval of 24h.

Group 5: Rats were pretreated with EWP (200mg/kg b.wt.) dissolved in saline for a period of 30 days

At the end of 16 weeks, experimental rats (n = 6 per group) were sacrificed. Blood was collected using sodium citrate as anticoagulant. The heart tissues were dissected out immediately and washed in ice-cold saline. Hundred milligrams of heart tissue was weighed accurately and homogenized by a Teflon® homogenizer in ice cold 0.1M Tris–HCl buffer, pH 7.2 and samples were stored at −80 ◦C for analytical procedures.

Assay of cardiac marker enzymes

The heart homogenate and serum were used to assay the cardiac specific markers like creatinine kinase (CK) [12], creatinine kinase – muscle brain (CK-MB) [13], troponin T (CnTn) (MSD 96 well MULTI-ARRAY), aspartate aminotransferase (AST) and alanine amino transferase (ALT) [14], alkaline phosphatase (ALP) [15], and lactate dehdrogenase (LDH) [16] respectively.

Histological examination

A portion of the heart was cut into two to three pieces of approximately 6 mm3 sizes and fixed in phosphate buffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 µm thickness of heart tissues were cut and stained with haematoxylin–eosin. The thin sections of heart were made into permanent slides and the method adopted for examination was [17]. The photomicrographs were shot using a Zeiss Axioscope 2 Plus photomicroscope at ×200 magnification.

Statistical analysis

Results are expressed as mean± S.D. One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Tukey's test using a statistical package program (SPSS 10.0 for Windows).

RESULTS

From the previously conducted acute toxicity study with selected doses of EWP (100, 200, and 300mg/kg b.wt) is devoid of toxic to the experimental rats [18]. Hence the present study was progressed to evaluate the protective effect of EWP against isoproterenol induced myocardial damage within the selected concentration of 200mg/kg b.wt.

(**Table 1 and 2**) represent the activities of marker enzymes (CK, CKMB, Troponin T, AST, ALT, ALP and LDH) in serum and heart tissues of control and experimental animals. There was a significant (P□0.05) rise in the levels of these diagnostics marker enzymes in the serum of group II, ISO administered rats as compared to that of group I (control) rats. These marker enzymes serve as sensitive indices to assess the severity of myocardial infarction. The administration of EWP (group III) showed a significant decrease in the activities of these enzymes in systemic circulation as compared with group II animals. At the same time the group V animals did not showed any adverse changes with the parameters tested.

Table 1: Effect of EWP on the activity of Ck, CK-MB and CnTn in serum and heart tissue of Isoproterenol Induced Rats

GROUPS	CK		CK-MB		Troponin T (cTnT)	
	Serum	Heart	Serum	Heart	Serum	Heart
Group I (Control)	125.16 ±0.78	226.22 ±0.25	39.41 ±1.26	14.66 ±0.33	115.77 ±0.01	88.14 ±0.05
Group II (ISO)	134.36 ±0.89[a]	195.22 ±0.54[a]	64.88 ±0.79[a]	28.24 ±0.21[a]	195.56 ±0.03[a]	89.76 ±0.05[a]
Group III (ISO+EWP)	120.30 ± 2.05[b]	202.48 ±0.40[b]	44.19 ±1.76[b]	19.36 ±0.32 [b]	94.32 ±0.08[b]	105.55 ±0.03[b]
Group IV (ISO+Atrovastatin)	126.45 ±2.33[c]	212.71 ±0.44[c]	49.67 ±2.01[c]	12.81 ±0.54[c]	91.06 ±0.07[c]	100.28 ±0.05[c]
Group V (EWP)	125.11 ±0.78	226.10 ±0.25	39.21 ±1.26	14.31 ±0.33	115.26 ±0.01	88.01 ±0.05

Units:
CK, CKMB: IU/l (Serum), µmol of phosphorus liberated/min/mg protein (Heart)
CnTn: IU/L (Serum), µmol of pyruvate liberated/min/mg protein (Heart)
Values are expressed as mean± SD for 6 animals in each group;
P values:
a<0.05 statistically significant when compared with group I
b and c <0.05 statistically significant when compared with group II

Table 2: Effect of EWP on the activity of AST, ALT, ALP and LDH in Serum and Heart Tissue of Isoproterenol Induced Rats

GROUPS	AST		ALT		ALP		LDH	
	Serum	Heart	Serum	Heart	Serum	Heart	Serum	Heart
Group I (Control)	125.01 ±0.42	145.28 ±0.62	17.53 ±0.36	137.64 ±0.37	113.95 ±0.0.45	124.15 ±0.47	93.98 ±0.0.44	68.00 ±0.46
Group II (ISO)	212.27 ±0.41[a]	94.16 ±0.54[a]	98.22 ±0.32[a]	78.60 ±0.29[a]	245.28 ±0.44[a]	205.28 ±0.46[a]	163.02 ±0.49[a]	54.50 ±0.43[a]
Group III (ISO+EWP)	183.68 ±0.33[b]	104.17 ±0.28[b]	77.37 ±0.46[b]	85.86 ±0.43[b]	165.71 ±0.37[b]	199.04 ±0.39[b]	91.42 ±0.34[b]	99.08 ±0.38[b]
Group IV (ISO+Atrovastatin)	134.33 ±0.42[c]	113.02 ±0.37[c]	59.00±0.43[c]	102.32 ±0.46[c]	130.44 ±0.42[c]	113.19 ±0.43[c]	103.68 ±0.32[c]	98.62 ±0.41[c]
Group V (EWP)	125.11 ±0.42	145.33 ±0.62	17.27 ±0.36	137.00 ±0.37	113.02 ±0.0.45	124.09 ±0.47	93.15 ±0.0.44	68.01 ±0.46

Units:
AST, ALT, ALP: IU/L (Serum), nmoles of phenol liberated /min / mg protein (Heart)
LDH: IU/L (Serum), nmoles of pyruvate liberated /min / mg protein (Heart)
Values are expressed as mean± SD for 6 animals in each group;
P values:
a<0.05 statistically significant when compared with group I
b and c <0.05 statistically significant when compared with group II

Histopathological examination of heart tissue under light microscope was performed to observe the effect of EWP on ISO induced rats, on the structural integrity of the cells of heart under the condition of myocardial infarction (**figure 1**).
Myocardium tissue sections taken from group control (group I) rat heart showed a normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. The pictogram of control group revealed a normal architecture with regular morphology of myocardial cell membrane (**figure 1A**). The heart sections of ISO (group II) treated pathogenic rats showed confluent necrosis of cardiac muscle fibre with infiltration of red blood cells leading to impairment of membrane structural and functional integrity (**figure 1B**). The tissue take from EWP treated rats (group III) showed the morphology of myocardium was essentially within the normal limits (**figure 1 C**). No area of necrosis, congested vessels and cellular infiltration was seen indicating cardioprotective effect of EWP at 200mg/kg b.wt. Atrovastatin treated rats (Group IV) showed normal morphology with absence of inflammation and sign of muscle necrosis (**figure 1D**).

DISCUSSION

Injury due to myocardial ischemia occurs following inhibition of the aerobic oxidation of glucose, augmentation of anaerobic glycolysis and accumulation of lactic acid dehydrogenase. Meanwhile, reduction of ATP production, disruption of ionic gradients and degradation of membrane stability can lead to out leakage of enzymes normally residing within cardiomyocytes. Consequently, the content of myocardial enzymes in blood serum increases, so

changes in serum myocardial enzymes are considered to be a measure of impairment produced by myocardial ischemia. [19]. In the present investigation the myocardial enzymes (CK, CKMB, Troponin T, AST, ALT, ALP and LDH) was found to get ameliorated and leaks from cardiac tissue into serum in ISO intoxicated rats (group II) which confirms the onset of myocardial necrosis. Hence the entire concentration of marker enzyme was found to be decreased in heart tissue of group II rats when compared to control. Furthermore, the amount of enzymes appearing in serum is proportional to the number of necrotic cells [20].

The creatinine kinase system plays an important role in myocardial energy metabolism by maintaining ADP levels high at the mitochondria, where ATP is generated and low at sites of ATP utilization. This is postulated to contribute to the maintenance of high free energy of ATP hydrolysis, thereby enhancing the efficiency of the energy utilization process. In addition, a CK shuttle has been proposed in which high energy phosphate transport within the cell is facilitated by the higher diffusibility of creatinine and phosphocreatine relative to ADP [21]. CK and more particularly its isoenzyme CK-MB still have a formal place in defining myocardial infarction. These enzymes normally exist in cellular compartment and leak out in to the plasma during myocardial injury due to disintegration of contractile elements and sarcoplasmic reticulum [22]. Troponin T is a protein found in cardiac tissue. When the myocardial damage occurs the cytosolic troponins reach the blood stream quickly resulting in a rapid peak of serum troponin [23]. When myocardial cells, containing cardiac enzymes like AST, ALT ALP and LDH is damaged or destroyed due to deficient oxygen supply or glucose, the cell membrane becomes permeable or may rupture, which results in the leakage of these enzymes. This accounts for the decreased activities of these enzymes in heart tissue of ISO-induced rats. This might be due to the damage caused by the β - agonist that has rendered it leaky [24].

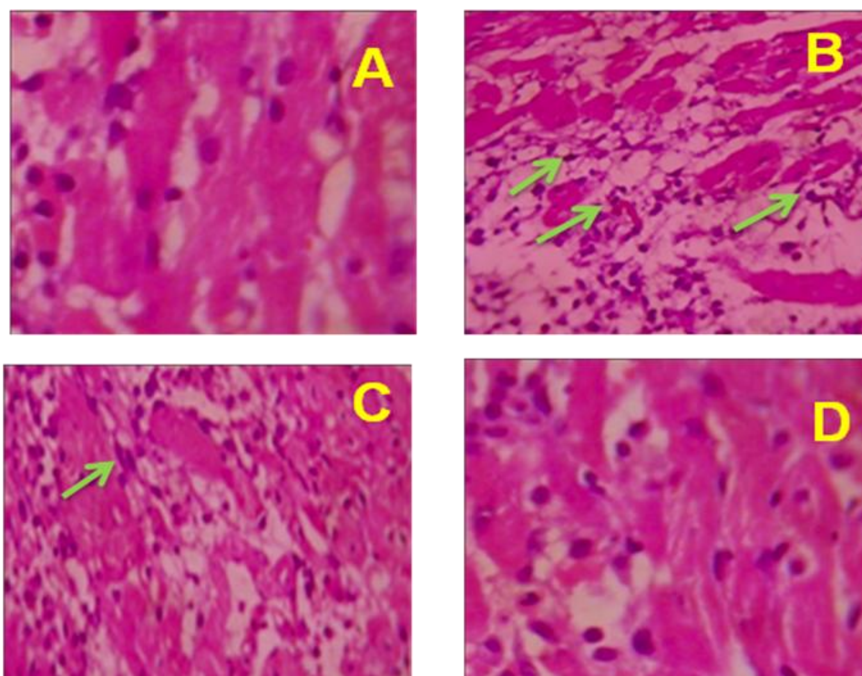

Figure 1: Histopathological examinations (A). Haematoxylin and eosin (×200) stained microscopic myocardium section of group I rats (control). Haematoxylin and eosin (×200) stained microscopic heart section of group II rat treated with ISO (B). (C) Rats orally pretreated with 200mg/kg b.wt. of EWP (group III). (D) Haematoxylin and eosin (×200) stained microscopic heart section of rats treated with atrovastatin, a standard drug.

In this situation where there is high activity of these enzymes in the blood due to oxidative damage caused by isoproterenol treatment, the administration of EWP ameliorate tissue dysfunction, since EWP are known to improve tissue integrity. Senthilkumar *et al.* [25] who stated that the activities of CK enzyme in serum were maintained at near normal levels in the rats received, pre-co-treated with garlic oil and Vitamin E respectively. Narin *et al.,* [26] who reported that there was an increase in the activity of troponin in serum of ISO treated rats and its protection by tryptophan. Our study was in accordance with previous reports of [27] that the treatments with earthworm extract as well as silymarin plus decreased the activity of AST, ALT indicating that earthworm extract may protect and accelerate the hepatocytes regeneration. Similarly, [28] found that serum ALT and AST levels increased markedly after the paracetamol administration and returned to its normal levels by treatment with earthworm extract [29]. The results indicate that EWP has the tendency to reduce the elevated cardiac marker enzymes proving its cardioprotective effect and also prolong the viability of myocardial cell membrane stabilizing action and proving its protective role.

Light microscopy of all the rat tissue sections treated with EWP showed a well-preserved normal morphology of cardiac muscle with no evidence of focal necrosis when compared to the ISO-induced heart, which showed the non-toxic nature of the EWP. Thus, from the biochemical and histological evidence it may be accepted that 200 mg kg^{-1} of EWP have protective nature against ISO-induced myocardial injury. Furthermore histopathological observations revealed that oral pretreatment with EWP at 200mg/kg b.wt. prevented the degeneration of myofibriller tissue and leucocytic infiltration in myocardial infarction.

CONCLUSION

The present study revealed that the pre-treatment with EWP were safe and highly effective in preventing cardiac dysfunction in myocardial infracted rats, possibly due to their membrane stabilizing property. Histopathological view of tissue section also supports the biochemical investigation. Thus it could be concluded that EWP protects experimental animals from myocardial infarction as revealed by the amelioration of biochemical markers and cardiac tissue damage without any adverse effect which merit further detailed studies to develop it as a cardioprotective drug.

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