



(RESEARCH ARTICLE)



In vivo cardioprotective effect of withacoagulin (20 β , 27-Dihydroxy-1-oxo-(22R)-witha-2, 5, 24-tetraenolide) in experimental myocardial infarction in albino rats.

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Abstract

The present study was designed to evaluate the *in vivo* effect of withacoagulin isolated from *Withania coagulans* fruits in preventing myocardial infarction in rats. Effect of withacoagulin was compared with Vitamin E, a known cardioprotective antioxidant. Male albino rats (120–150 g) were divided into five experimental groups (n=8): Normal control, Toxic (isoprenaline) control, withacoagulin (25 mg/ kg) *per se*, Vitamin E control (100 mg/kg, p.o.) and withacoagulin treatment group (25 mg/kg; p.o.). After treatment of 28 days to the animals of withacoagulin and vitamin E group, the rats in the isoprenaline toxic control and withacoagulin/vitamin E treatment group were given isoprenaline (85 mg/kg; s.c.) on day 29 and day 30, at an interval of 24 hour to induce myocardial infarction. On day 31st, the animals were euthanized using CO₂ chamber and hearts were removed for histopathological and biochemical studies. Biochemical assessment showed significant decrease (P<0.05) in glutathione level. Highly significant decrease (P<0.01) in the level of superoxide dismutase, catalase, creatinine phosphokinase and lactate dehydrogenase has been observed. A highly significant increase (P<0.01) in lipid peroxidation marker malonyldialdehyde level was observed in the hearts of isoprenaline control rat as compared to normal control rat. On histopathological examination, myocardial damage was further confirmed due to injected isoprenaline. Withacoagulin (25 mg/ kg) showed a strong cardioprotective effect in isoprenaline-induced myonecrosis in rat. Augmentation of endogenous antioxidants and maintenance of the myocardial antioxidant status may contribute to its cardioprotective effect.

Keywords: Withacoagulin; *Withania coagulans*; Vitamin E; Isoprenaline; Myocardial infarction; Cardioprotection.

1. Introduction

Myocardial infarction (MI) is highly lethal cardiovascular disorder and has been a topic for intense investigation [1]. Acute myocardial infarction (AMI) is characterized by a focus of necrosis resulting from low tissue perfusion showing coagulated necrosis (White infarct formation), a characteristic of myocardial infarction [2]. The pathological changes in MI are characterized by vacuolization and myocytolysis followed by necrosis (irreversible cell injury) and edema (Na⁺-H₂O entry). Within 1-3 days macrophages causes phagocytosis and yellow tan is observed. Troponin protein starts leaking from cardiac muscles into the blood which can be determined biochemically.

Isoprenaline [1-(3, 4-dihydroxyphenyl)-2-isopropylamino ethanol hydrochloride] (ISO), a synthetic catecholamine is a β -adrenergic agonist and develops an infarct-like necrosis in the heart muscle caused by severe oxidative stress in the myocardium. Due to generation of free radicals and stimulation of lipid peroxidation isoprenaline may cause irreversible damage to the myocardial membrane [4]. The isoprenaline treated albino rats have been used to affect the adenylate cyclase cAMP signaling pathway in the myocardium [5, 6]. The albino rats developed myocardial necrosis and a progressive enlargement of the left ventricular cavity out of proportion to mass due to isoprenaline, as in humans with

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discrete myocardial infarction [7]. The albino rats develop cardiomyopathy and a functional desensitization of β -adrenoceptor function of heart [8].

Withania coagulans Dunal (family *Solanaceae*) is widely used in indigenous system of medicine. It is distributed in the east of the Mediterranean region extending to South Asia including northern and western India [9]. The drug has been reported to possess anti-inflammatory [10], cardiogenic activities [11], hepatoprotective [12], antifungal [13], free radical scavenging activity [14], hypoglycemic, hypolipidemic [15], wound healing activity [16] and useful in diabetic nephropathy [17]. The aqueous extract of *W. coagulans* fruit is diuretic which may be associated with the presence of active principles of polar nature where withanolides are the main chemical protagonist for this activity [18]. *W. coagulans* has a wide range of active phytoconstituents mainly withanolides (steroidal lactones), withaferin A, and coagulins [19, 20, 21]. Withacoagulins are steroidal lactones isolated from *Withania coagulans* fruits [22].

The animal model of myocardial infarction (MI) plays an important role in understanding the prevention, diagnosis, and therapy of human myocardial infarction (Heart attack). In the present scenario of therapy it has been realized that phytoconstituents can influence the course of cardiovascular diseases and its treatment by providing an integrated structure of nutritional and therapeutic substances which aid in restoring and maintaining homeostasis of the physiological system [3]. Current modern therapy has more side effects, high mortality and is costlier. Hence evidence based therapies due to pure phytoconstituents may provide better result in survival rate and undoubtedly are cheaper to treat cardiovascular disorders like myocardial infarction.

2. Material and methods

Isoprenaline (Sigma Chemical Company) was a generous gift from Department of Pharmacology, AIIMS, New Delhi. All other chemicals and reagents were of analytical grade.

2.1. Isolation scheme of withacoagulin from *Withania coagulans* berries [22]

Withacoagulin was isolated from *W. coagulans* fruits purchased from local spice and herbs market in Delhi. The fruits were identified and authenticated by National Herbarium of cultivated plants (NHCP), National Bureau of Plant Genetic Resources, New Delhi and the specimen voucher (NHCP/NBPGR/2014-09) has been retained. The fruits were dried, weighed and powdered for extraction and isolation of withacoagulin.

The *Withania coagulans* berries were shade dried and crushed. The powdered berries were macerated in a mixture of chloroform and ethanol (1:1) for three days by occasional shaking. The mixture was filtered and dried in rotary evaporator. A semisolid mass so obtained after drying was fractionated by solvent-solvent extraction. Crude extract so obtained was suspended in hot water and extracted three times with n-hexane using a separating funnel. Aqueous layer was separated and extracted with ethyl acetate. The organic layer was separated and dried in rotary evaporator at 35°C and ethyl acetate fraction (WCE) was obtained. WCE was subjected to normal phase column chromatography (silica gel 60, 230-400 mesh) using the mobile phase n-Hex: EA/5:1-0:1. Multiple fractions (100ml each) were obtained and combined followed by column chromatography (sephadex LH20) using ethanol as mobile phase. Later few fractions were combined and submitted to normal phase column chromatography (silica gel 60, 5-40 μ m) using the mobile phase n-Hex: EA/5:1-0:1. Finally few more fractions were collected and combined. They were subjected to RP-MPLC (Bondesil-C18, 40 μ m) by using the gradient mobile phase EtOH:H₂O/30:70. The collected fractions were dried to get withacoagulin. The obtained isolate was confirmed spectroscopically as withacoagulin.

2.2. Formulation and administration of withacoagulin

Withacoagulin was suspended in 0.5% carboxy methylcellulose (CMC). Each animal from two different groups received 1.0 ml of withacoagulin suspension (25 mg/kg; p.o.) daily.

2.3. Induction of myocardial infarction

After treatment of 28 days to the animals of withacoagulin and vitamin E group, the albino rats in the toxic control and withacoagulin/vitamin E treatment groups were given isoprenaline (85 mg/kg body weight, dissolved in physiological saline), subcutaneously on day 29 and day 30, at an interval of 24 hours.

2.4. Experimental animals

Forty healthy male albino Wistar rats (120-180 g), eight animals for each group were selected for the study. The animals were housed in polypropylene cages (four in each cage) with rice husk bedding, standard pellet diet (Hindustan Lever

Limited, Chandigarh) and water bottle to provide drinking water *ad libitum*. Standard laboratory condition of temperature (25 °C, humidity (50%) and dark- light cycle of 12 hours each were maintained inside animal house. The study was approved by the Institutional Animal Ethics Committee (RIVTE/ IAEC/16/02) of Ram-Eesh institute of vocational and technical education, Greater Noida (UP), India.

2.5. Treatment protocol

The rats were divided into five groups having eight animals in each group. The rats in Group I (Normal Control) were given 1.0 ml of 0.5% carboxy methyl cellulose (CMC) p.o. every day. The rats in Group II were given isoprenaline (85 mg/kg body weight) subcutaneously on day 29th and day 30th at an interval of 24 hours. The rats of Group III were given withacoagulin (25 mg/kg; p.o.) for a period of 30 days. Group IV and Group V rats were given withacoagulin and Vitamin E respectively for 30 days and at the end of the experimental period on 29th and 30th days the rats were given isoprenaline (85 mg/kg body weight) injections subcutaneously twice at an interval of 24 hours. On day 31st, after an overnight fast the animals were euthanized using CO₂ chamber followed by decapitation. The hearts were removed and processed for histopathological and biochemical studies. For performing biochemical estimations, sliced hearts were stored in liquid nitrogen until further analysis.

2.6. Biochemical studies

A 10% homogenate of myocardial tissue was prepared in 50 mM phosphate buffer, pH 7.4, and an aliquot was used for the assay of malonyldialdehyde [23]. The homogenate was centrifuged at 7000 rpm for 15 minutes and the supernatant was used for the estimation of biochemical parameters: lactate dehydrogenase [24], glutathione [25], glutathione peroxidase [26], superoxide dismutase [27], catalase [28] and protein [29]. Creatinine phosphokinase was estimated spectrophotometrically using a kit from Randox Laboratories, USA [30].

2.7. Histopathological studies

At the end of the experiment, after euthanizing the rats in CO₂ chamber followed by decapitation the hearts were removed. The hearts were sliced for immediate fixation of myocardial tissues in 10% buffered neutral formalin solution. The fixed tissues were embedded into paraffin and serial sections were cut.

2.8. Statistical analysis

Descriptive statistics mean and standard deviation were calculated for all variables of each group. In the isoprenaline group, one-way Analysis of Variance (ANOVA) was applied for statistical analysis with post-hoc analysis (Bonferroni Multiple Range Test) and P value <0.05 has been considered as statistical significance level.

3. Results and discussion

A significant increase and restoration in the glutathione (GSH) level ($P < 0.05$) was observed in withacoagulin (25 mg/kg) treated groups as compared to isoprenaline (ISO) toxic group (Table 1). Withacoagulin (25 mg/kg) treatment *per se* has highly significant augmentation on endogenous antioxidant enzymes superoxide dismutase ($P < 0.01$, Table 2) and glutathione peroxidase ($P < 0.01$). Along with this, a significant increase in the myocardial catalase activity in withacoagulin (25 mg/kg) ($P < 0.05$) groups were also observed (Table 2). Isoprenaline-induced myocardial necrosis resulted in a significant depletion of antioxidant enzymes: catalase ($P < 0.05$) Table 2), superoxide dismutase ($P < 0.05$, Table 2) compared to normal control.

We observed that the malonyldialdehyde levels were significantly elevated ($P < 0.05$) in the isoprenaline control group (Table 2). Withacoagulin (25 mg/kg) treatment significantly inhibited lipid peroxidation ($P < 0.05$) and preserved membrane integrity. A fall in myocardial enzymes creatinine phosphokinase ($P < 0.01$, Table 3), lactate dehydrogenase ($P < 0.01$, Table 3) was observed in the withacoagulin group compared to ISO toxic control group.

Table 1 Serum endogenous glutathione level (Antioxidant parameter)

Groups (n=8)	GSH ($\mu\text{mol GSH}/\text{mg prot}$)	GPx ($\text{nmol CDNB}/\text{minute}/\text{mg prot}$)	GR ($\text{nmol NADPH}/\text{minute}/\text{mg prot}$)
Gp I Normal Control	1.37 ± 0.13	5.67 ± 0.31	6.37 ± 0.29
Gp II ISO (85 mg/kg)	0.21 ± 0.18	1.79 ± 0.21	1.31 ± 0.18
Gp III WC (25 mg/kg) per se	1.22 ± 0.15	5.30 ± 0.29	5.14 ± 0.53
Gp IV WC (25 mg/kg) + ISO	1.08 ± 0.10	3.87 ± 0.25	3.81 ± 0.32
Gp V Vit E + ISO	0.98 ± 0.07	3.34 ± 0.18	3.57 ± 0.45

Table 2 Antioxidant enzyme activity

Groups (n=8)	SOD (Unit/g of tissue)	Catalase ($\mu\text{mol of H}_2\text{O}_2$ consumed/min/g of tissue)	TBARS ($\text{nmol MDA/g of tissue}$)
Gp I Normal Control	13.55 ± 0.62	2.66 ± 0.24	1.65 ± 0.12
Gp II ISO (85 mg/kg)	5.38 ± 0.31	0.54 ± 0.02	3.82 ± 0.21
Gp III WC (25 mg/kg) per se	12.56 ± 0.61	1.87 ± 0.37	2.23 ± 0.16
Gp IV WC (25 mg/kg) + ISO	12.14 ± 0.33	2.09 ± 0.32	2.41 ± 0.21
Gp V Vit E + ISO	11.16 ± 0.31	2.22 ± 0.35	1.53 ± 0.21

Table 3 Cardiac Injury Markers

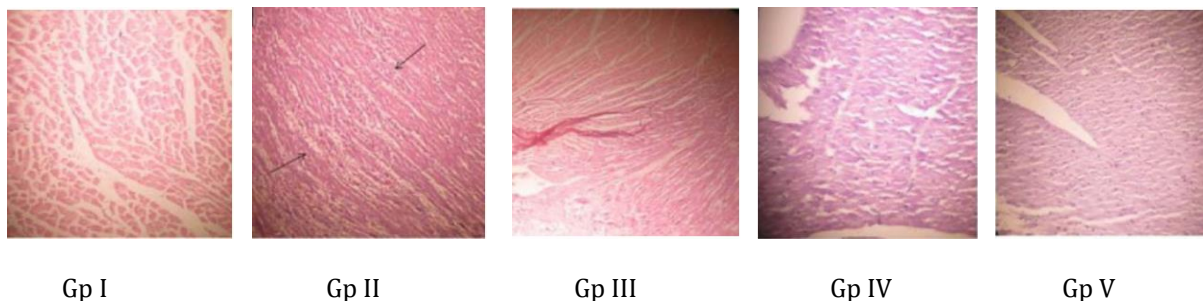
Groups (n=8)	LDH (IU/I)	CK-MB (IU/I)	AST (IU/I)	ALT (IU/I)	Troponin-I (ng/ml)
Gp I Normal Control	363.12 ± 32.61	483.33 ± 36.19	249.45 ± 7.21	77.14 ± 2.93	0.16 ± 0.04
Gp II Isoprenaline (85 mg/kg)	1315 ± 43.22	1923.57 ± 46.09	308.31 ± 7.42	176.85 ± 3.14	1.34 ± 0.22
Gp III WC (25 mg/kg) per se	389.27 ± 40.89	523.54 ± 45.60	264.39 ± 7.11	88.12 ± 2.96	0.26 ± 0.06
Gp IV WC (25 mg/kg) + ISO	967 ± 45.87	1376.87 ± 42.34	279.81 ± 7.57	1.30 ± 3.02	0.87 ± 0.13
Gp V Vit E + ISO	1077 ± 48.45	1658.74 ± 45.10	285.41 ± 8.22	132.47 ± 2.80	1.05 ± 0.11

Table 4 Oxidative stress markers

Groups (n=8)	Oedema	Infiltration	Necrosis
Gp I Normal Control	0 ± 0	0 ± 0	0 ± 0
Gp II Isoprenaline (85 mg/kg)	2.46 ± 0.47	2.47 ± 0.49	2.82 ± 0.31
Gp III WC (25 mg/kg) per se	0.31 ± 0.18	0.23 ± 0.14	0.50 ± 0.10
Gp IV WC (25 mg/kg) + ISO	0.81 ± 0.46	0.86 ± 0.21	0.87 ± 0.24
Gp V Vit E + ISO	0.45 ± 0.21	0.32 ± 0.25	0.53 ± 0.21

3.1. Histopathological assessment of injury

Histopathological examination showed a significant myocardial membrane damage and infiltration of inflammatory cells in the isoprenaline control group as compared to normal control group. Moreover extensive myonecrosis with fibroblastic proliferation and presence of chronic inflammatory cells were observed in the isoprenaline control group compared to that of normal. There is highly significant percentage myofiber loss due to necrosis in the isoprenaline control group was ($P < 0.001$, Table 4) as compared to normal control. In the present study, *withacoagulin* (25 mg/kg) and vitamin E (100 mg/kg) treatment significantly prevented myonecrosis as indicated by significant reduction in the infiltration of inflammatory cells, vacuolar changes as well as oedema as compared to the isoprenaline control group. There was significantly less % fiber loss in the vitamin E-treated group ($P < 0.05$). But it was shown that there is highly significant decrease ($P < 0.001$) in the % fibre loss in the *withacoagulin* (25 mg/kg) groups as compared to isoprenaline control.



*Arrows indicate infiltration and necrosis of the myocardium.

Figure 1 Histopathology of myocardial tissues: Gp I (Normal Control), Gp II (Isoprenaline Toxic), Gp III (WC *per se*), Gp IV (WC+ ISO), Gp V (Vit E + ISO)].

4. Conclusion

The present study was designed to evaluate the efficacy of *withacoagulin* isolated from *Withania coagulans* fruits in isoprenaline- induced myonecrosis in albino rats. The effect of *withacoagulin* on modulation of biochemical parameters like endogenous antioxidant glutathione, antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, lipid peroxidation product malonyldialdehyde and myocardial enzymes lactate dehydrogenase and creatinine phosphokinase have been studied. Increase in malonyldialdehyde level was observed in the heart tissue after isoprenaline administration. In addition, isoprenaline administration decreased the reduced glutathione content as well as the antioxidant enzyme activity (superoxide dismutase, catalase and glutathione peroxidase) in cardiac tissues. A significant depletion of lactate dehydrogenase and creatinine phosphokinase, an important marker of myocardial injury, in the isoprenaline group was observed. The observation that vitamin E and *withacoagulin* treatment significantly restored lactate dehydrogenase and creatine phosphokinase activity compared to the isoprenaline control group was suggestive of their cardioprotective effect. Both these drug treatments restored the myocardial antioxidant status and maintained membrane integrity as evidenced by a decline in malonyldialdehyde level. Furthermore, histopathological examination confirmed the cardioprotective effect of *withacoagulin* in albino rats as evidenced by the presence of focal myonecrosis with myophagocytosis and lymphocytic infiltration (myocarditis) in the subendocardial region.

In summary, the present study strongly suggests that multiple mechanisms may be responsible for the cardioprotective effect of *withacoagulin*.

Compliance with ethical standards

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Disclosure of conflict of interest

The author(s) have no conflicts of interest to disclose with anyone else.

Statement of ethical approval

The study was approved by the Institutional Animal Ethics Committee (RIVTE/ IAEC/16/02) of Ram-Eesh Institute of Vocational and Technical Education, Greater Noida, Uttar Pradesh, India (Animal House registration number: 385/PO/Re/S/01/CPCSEA).

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