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"EVALUATION OF CARDIOPROTECTIVE EFFECT OF *COW URINE ARK* ON ISOPRENALINE INDUCED MYOCARDIAL INJURY IN *WISTAR* ALBINO RATS"

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ABSTRACT

The present study was conducted to evaluate the cardioprotective effect of *Cow urine ark* (*CUA)* by isoprenaline-induced myocardial ischemia in male *Wistar* albino rats. Acute toxicity study was performed. Chemical analysis of *CUA* contents was carried out. For present study, total 72 rats were randomized into 6 groups: normal saline, isoprenaline control, *CUA*, Low dose (1.4 ml/kg) and High dose (2.8 ml/kg) *CUA* and Carvedilol (1 mg/kg) group. Myocardial injury was produced by subcutaneous injection of isoprenaline (85 mg/kg) on day 27th and 28th. ECG changes, cardiac injury markers, antioxidant markers and histopathological changes were observed. Study results show decrease in troponin - I levels in Low and High dose *CUA* group as compared to Isoprenaline control group and histopathological changes show statistically significant cardioprotective effect with high dose *CUA*. So we can conclude that high dose *CUA* shows the cardioprotective effect but further study with higher doses is necessary.

KEYWORDS: Cardioprotective; Carvedilol; *CUA*; Isoprenaline; Myocardial injury.

INTRODUCTION

Major blood supply to the myocardium is derived from coronary arteries. When coronary arteries are blocked due to thrombosis it produces ischemia or necrosis of the affected part of the myocardium. During myocardial ischemia, there is an increase in reactive oxygen species (ROS) like hydroxyl radicals (OH^-) and superoxide anion (O_2^-) in ischemic tissues.[1] These induce the oxidative stress, resulting in oxidative damage to the membrane.[2,3] In addition, development of lipid peroxides, damaged antioxidant defense system and associated inflammatory response leads to metabolic derangement. [4] There is the release of inflammatory cytokines which along with ROS play a crucial role in intracellular Ca^{2+} overload which eventually leads to necrotic changes.[5] Ischemic heart disease is the leading cause of death in the world.[6, 7] It is responsible for the largest proportion of non-communicable deaths (47.9 per cent). Ischemic heart disease accounts for 1.2 million deaths in India. It accounts for about 40 percent of Disease Associated Life Years (DALYs) lost.[8]

Cow urine is found to have many beneficial effects like antioxidant (Responsible for Hepatoprotective effect)[9, 10, 11, 12] renoprotective and antidiabetic effect.[13]

Following are few important elements found in cow's urine have beneficial action:
Cardiovascular health is maintained by kallikrein which is a vasodilator and enzyme urokinase which acts as a fibrinolytic agent;[14] Nitrogen, uric acid, phosphates and hippuric acid act as diuretic agents and maintain renal health.

So, from above mentioned observations, antioxidants, vasodilator and fibrinolytic properties may possibly offer cardioprotection in the event of ischemic myocardial injury. So, the present study was planned to explore the protective effects of *CUA* on myocardial ischemia.

MATERIALS AND METHODS

All experiments were carried out after prior permission from Institutional Animal Ethics Committee (IAEC) (Registration number and date of registration, 577/Go/C/02/CPCSEA dated 17th November, 2011), Government Medical College, Bhavnagar, Gujarat, India. (IAEC Protocol No. 38/2015, dated: 29/03/2015).

Experimental animals

Male *Wistar* albino rats (260 ± 100 g) were procured from the central animal house of the institution. Rats were housed in standard transparent polypropylene cages with wheat husk bedding, changed every 24 hour and rabbits were housed in stainless steel standard size cages. Animals were kept under controlled room temperature and humidity (26 ± 3°C; 40 ± 5%) in a 12-hour light-dark cycle. Animals were acclimatized to laboratory conditions for one week prior to starting the experiment. The rats were given standard diet and water *ad libitum*.

Chemicals and reagents

CUA was procured from Parthvimeda Gau Pharma Pvt. Ltd., Rajasthan, India. Isoprenaline, gallic acid, quercetin and urethane were obtained from the Sigma Chemical Company, St. Louis, MO, USA. Carvedilol was purchased from Selleck chemicals, Munich, Germany. Isoprenaline solution was freshly prepared in normal saline at the time of injection.

Determination of total phenols and flavonoids

Total phenolic content was determined according to Folin-Ciocalteu method and flavonoid content in the *CUA* was determined by a colorimetric method.[15]

Acute toxicity study

It was conducted by using female *Swiss* albino mice (20-45 g) in accordance with Organization for Economic Co-operation and Development (OECD) guideline No. 423 for *CUA*. The animals were observed initially for toxic manifestations and then for any disability or death for 14 days.

Experimental groups:

Total 72 male *Wistar* albino rats (Group = 6, n = 12), were randomly allocated into each group by randomization using Rando software [Version 1.2 (c) R. Raveendran, 2004]. Myocardial Infarction was induced in rats by giving isoprenaline (85 mg/kg) subcutaneously (s. c.) on day 27th and 28th at the 24-hour interval.[16] At the end, animals were sacrificed and 6 rats were used for antioxidant and 6 rats were used for histopathology study.

Distribution of study groups was as follow:

Group 1-Vehicle Control: Rats were given normal saline (1 ml/kg) orally for 28 days.

Group 2-Isoprenaline Control: Rats were given normal saline (1 ml/kg) orally for 28 days and isoprenaline (85 mg/kg) subcutaneously (s. c.) on 27th and 28th day.

Group 3-*CUA*: Rats were given *CUA* (1.4 ml/kg) orally for 28 days.

Group 4-Low dose *CUA*: Rats were given Low dose (1.4 ml/kg) *CUA* orally for 28 days and isoprenaline (85 mg/kg) s. c. on the 27th and 28th day.

Group 5-High dose *CUA*: Rats were given High dose (2.8 ml/kg) *CUA* orally for 28 days and isoprenaline (85 mg/kg) s. c. on the 27th and 28th day.

Group 6-Carvedilol: Rats were given carvedilol (1 mg/kg) orally for 28 days and isoprenaline (85 mg/kg) s. c. on the 27th and 28th day.

After administration of isoprenaline, the following parameters were recorded to evaluate the cardioprotective effect.

Electrocardiography recording

At the end of 28 days, 48 hours after the first dose of isoprenaline, all the animals were anesthetized by urethane (125 mg/100 g, i. p.). [17] ECG has recorded with the help of student's 2 channel physiograph and EKG coupler [Inco Ambala Co, Haryana, India; Paper speed - 50 mm/sec, sensitivity - 200 µV/cm, gain - maximum]. The electrodes made up from 26 gauge hypodermic needle were attached to both front and hind paw. One precordial lead was used and was placed in a position corresponding to V_4 in human. Heart rate, QRS interval, QT interval, R-R interval and Corrected QT interval (QTc) were calculated and compared between the groups.

Biochemical estimations

After recording of ECG, blood was collected through retro-orbital plexus using the glass micro-capillary tube. The serum was separated by centrifugation and used for the estimation of lactate dehydrogenase (LDH), creatinine kinase-MB (CK-MB), Troponin-I, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and serum uric acid. After blood collection, rats were sacrificed and heart was dissected out with mid abdominal incision. It was blotted in the filter paper, weighed and further processed for 6 rats for each, histopathological and antioxidant analyses.

Antioxidant estimation in heart tissue homogenate preparation

Hearts were washed with ice-cold saline followed by in 0.25 M sucrose solution and were finely sliced. Homogenate was prepared in chilled tris-HCl buffer solution [(10% w/v), (0.1 M), pH 7.4]. The homogenate was then centrifuged at 5000 rotations per minute (rpm) at 4°C using compact high speed refrigerated centrifuge (Kubota 6500, Japan). The clear supernatant was

obtained and then used for the estimation of superoxide dismutase (SOD) [18], catalase (CAT) by Hugo e. Aebi method (1984) and the marker of lipid peroxidation or malandialdehyde (MDA).[19]

Body weight was the weight of animal on the day of sacrification. Heart weight was measured after washing it in ice-cold saline after removal from the body, squeezing out the blood and blotted on the filter paper. Heart weight to body weight ratio was calculated in each group.

Histopathological analysis

6 hearts from each present study groups were preserved in 10% v/v formaldehyde, processed and embedded in paraffin wax. 5-6 mm thick sections were cut and stained with Haematoxylin and Eosin (H & E) stain and observed with a light microscope to evaluate the myocardial injury. All slides were coded, blinded and then analyzed by the trained pathologist from our institute. Histopathological observations for edema, infiltration and necrosis were categorized into 4 different grades ranging from grade 0 to 3. Grade 0: No change, 1: Focal changes, 2: Intermediate between 1 and 3, 3: Diffuse extensive changes.[20]

Statistical analysis

All the values were expressed as Mean ± SEM. Data were checked for normal distribution using the Kolmogorov-Smirnov test. Outliers were detected by an interquartile range in Microsoft excel and replaced by subsequent values and SigmaStat version 3.5 used for analysis.

RESULT

Antioxidant evaluation of *CUA*: Total phenolic content: 0.11 mg/L, Total flavonoid content: 0.19 mg/L.

Acute toxicity study: It was carried out on female S*wiss* albino mice with a dose of 1, 5, 10 and 20 ml/kg of *CUA* according to OECD guideline no. 423. No acute toxic effects were observed at the doses up to 20 ml/kg of *CUA*. Accordingly, $1/10^{th}$ [2 ml/kg, per orally (p. o.), for mice] dose of 1.4 ml/kg for rat was obtained by conversion factor 7. Hence, 1.4 ml/kg (Low dose) and 2.8 ml/kg (High dose) of *CUA* were selected for the screening of cardioprotective activity in the present study.

Effect of *CUA* on body weight, heart weight and heart weight/body weight ratio: In *CUA* group and high dose *CUA* group there was a significant increase in body weight on the 28^{th} day ($P<0.001$) as compared to day 1. There is a significant decrease in heart weight/body weight ratio in High dose *CUA* group ($P<0.01$) as compared to Isoprenaline control group.

Effect of *CUA* on cardiac injury markers: As shown in Table 1.

Effect *CUA* on electrocardiogram parameters: Heart rate is significantly increased in isoprenaline control group ($P<0.05$) as compared to vehicle control group. R-R Interval is significantly decreased in *CUA* group ($P<0.05$) as compared to vehicle control group.

Effect of *CUA* on biomarkers of oxidative stress

Superoxide dismutase (SOD): It was significantly decreased in isoprenaline control group ($P<0.01$) and in *CUA* group ($P<0.01$) as compared to vehicle control group. It is significantly decreased in *CUA* group as compared to active control group ($P<0.05$).

Lipid Peroxidase and Reduced Glutathione (GSH): They were significantly increased in isoprenaline control group ($P<0.05$, $P<0.05$) and in *CUA* group ($P<0.001$, $P<0.001$) as compared to vehicle control group.
Catalase: It was significantly increased in the active control group ($P<0.05$) as compared to isoprenaline control group.
Effect of *CUA* on histopathological changes in rats' heart: As shown in Table 2 & 3.

Table 1: Comparison of cardiac injury markers: Serum Troponin-I, LDH, CK-MB, SGOT, SGPT and Serum uric acid level.

Groups	Troponin-I (ng/ml)	LDH (IU/L)	CK-MB (IU/L)	SGOT (IU/L)	SGPT (IU/L)	S. Uric acid (mg/dl)
Group 1 - Vehicle Control	0.15 ± 0.04**	1676.5 ± 109.69$^{\#\#}$	433.08 ± 110.64$^{\text{ʊʊ}}$	353.5 ± 34.54	135.5 ± 22.17	1.78 ± 0.15$^{\Phi}$
Group 2 - Isoprenaline Control	1.52 ± 0.30	2090.00 ± 339.20$^{\#}$	1583.25 ± 284.67$^{\&\text{ʊ}}$	393.58 ± 48.28	108.08 ± 11.88	2.27 ± 0.10
Group 3 – *CUA*	0.10 ± 0.01***$^{\chi\chi}$	4194.42 ± 12.56**	2024.67 ± 250.73$^{\&\&\&\$}$	376.42 ± 58.01	226.00 ± 47.78	2.81 ± 0.34$^{@@\S}$
Group 4 - Low dose *CUA* 1.4 ml/kg	1.38 ± 0.38$^{\#\#\#\mu\chi}$	1874.25 ± 164.19$^{\#}$	1551.00 ± 389.67	336.00 ± 21.18	111.17 ± 11.52	1.43 ± 0.13$^{@\sigma\sigma\sigma}$
Group 5 - High dose *CUA* 2.8 ml/kg	0.67 ± 0.22$^{\#\mu}$	3434.67 ± 691.64	1938.67 ± 264.15$^{\&\&\&}$	423.92 ± 79.28	166.25 ± 37.02	1.59 ± 0.17$^{\sigma\sigma\sigma\Phi}$
Group 6 - Active Control (Carvedilol)	0.22 ± 0.03	1931.00 ± 234.59$^{\#}$	766.42 ± 143.36	279.83 ± 43.59	144.25 ± 23.87	1.83 ± 0.19$^{\sigma\sigma£}$
P value	< 0.0001	0.0001	< 0.0001	0.4437	0.5404	< 0.0001

Data is expressed as Mean ± SEM, *CUA = Cow urine ark* (n = 12 for each group).
** $P<0.01$ or *** $P<0.001$ as compared to Isoprenaline control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{\#}$ $P<0.05$ or $^{\#\#}$ $P<0.01$ or $^{\#\#\#}$ $P<0.001$ as compared to *CUA* group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{\&}$ $P<0.05$ or $^{\&\&\&}$ $P<0.001$ as compared to vehicle control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{\$}$ $P<0.05$ as compared to Active control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{@}$ $P<0.05$ as compared to Isoprenaline control group by One-way ANOVA followed by Tukey-Kramer Multiple Comparison test, $^{@@}$ $P<0.01$ as compared to vehicle control group by One-way ANOVA followed by Tukey-Kramer Multiple Comparison test, $^{\sigma\sigma}$ $P<0.01$ or $^{\sigma\sigma\sigma}$ $P<0.001$ as compared to *CUA* group by One-way ANOVA followed by Tukey-Kramer Multiple Comparison test. $^{\mu}$ $P<0.05$ as compared to vehicle control group by Mann-Whitney Rank Sum test, $^{\chi}$ $P<0.05$ or $^{\chi\chi}$ $P<0.01$ as compared to Active control group by Mann-Whitney Rank Sum test, $^{\text{ʊ}}$ $P<0.05$ as compared to Active control group by unpaired t-test $^{\Theta}$ $P<0.05$ as compared to Active control group by Mann-Whitney Rank Sum test § $P<0.05$ as compared to *CUA* group by Mann-Whitney Rank Sum test, $^{£}$ $P<0.05$ as compared to *CUA* group by unpaired t-test Test, $^{\Phi}$ $P<0.05$ as compared to Isoprenaline control group by Mann-Whitney Rank Sum test.

Table 2: Comparison of the histopathological score (Anterior aspect of heart wall).

Groups	Edema	Infiltration	Necrosis
Group 1 - Vehicle Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00$^{\mu}$
Group 2 - Isoprenaline Control	0.5 ± 0.34	0.67 ± 0.42	1.17 ± 0.37*
Group 3 - *CUA*	0.00 ± 0.00$^{\#}$	0.00 ± 0.00$^{\#}$	0.00 ± 0.00$^{@}$
Group 4 - Low dose *CUA* 1.4 ml/kg	0.67 ± 0.21*	1.17 ± 0.31*	0.67 ± 0.21
Group 5 - High dose *CUA* 2.8 ml/kg	0.00 ± 0.00$^{\#}$	0.33 ± 0.21	0.17 ± 0.17$^{\mu}$
Group 6 - Active Control (Carvedilol)	0.00 ± 0.00$^{\#}$	0.33 ± 0.33	0.17 ± 0.17$^{\mu}$
P value	0.0075	0.0242	0.0027

Data is expressed as Mean ± SEM, *CUA= Cow urine ark* (n = 6 for each group).
* *P*<0.05 as compared to vehicle control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{\#}$ *P*<0.05 as compared to Low dose *CUA* group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{@}$ *P*<0.05 as compared to Isoprenaline control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test. $^{\mu}$ *P*<0.05 as compared to Isoprenaline control group by Mann-Whitney Rank Sum Test.

Table 3: Comparison of the histopathological score (Posterior aspect of heart wall).

Groups	Edema	Infiltration	Necrosis
Group 1 - Vehicle Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Group 2 - Isoprenaline Control	0.67 ± 0.33	0.67 ± 0.33	1.33 ± 0.21**
Group 3 - *CUA*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00$^{\#\#}$
Group 4 - Low dose *CUA* 1.4 ml/kg	0.50 ± 0.34	0.83 ± 0.48	0.10 ± 0.37
Group 5 - High dose *CUA* 2.8 ml/kg	0.33 ± 0.21	0.83 ± 0.31	0.33 ± 0.21
Group 6 - Active Control (Carvedilol)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00$^{\#\#}$
P value	0.1040	0.0186	0.0003

Data are expressed as Mean ± SEM, *CUA = Cow urine ark* (n = 6 for each group).
** *P*<0.01 as compared to vehicle control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $^{\#\#}$ *P*<0.01 as compared to Isoprenaline control group by Kruskal-Wallis test followed by Dunn's Multiple Comparison tests.

DISCUSSION

Effect of *CUA* on body weight, heart weight and heart weight / body weight ratio: In isoprenaline control group, there was marked increased in heart weight and heart weight/ body weight ratio as compared to vehicle control group, *CUA* group, high dose *CUA* group and active control group which might be attributed due to increased water content, edematous intramuscular space and increased protein content.[21]

Effect of *CUA* on cardiac injury markers: There was a dose-dependent decrease in Troponin-I levels in both low dose and high dose *CUA* group as compared to isoprenaline control group which did not reach the statistical significance. It was significantly decreased in *CUA* group as compared to isoprenaline control group (*P*<0.001). There was an increase in LDH, CK-MB and serum uric acid level in *CUA* group.

Serum uric acid: It was significantly increased in isoprenaline control group as compared to low dose *CUA* group and high dose *CUA* group which can be due to proteolysis and excessive degradation of purine nucleotides.[22] (Table 2).

In *CUA* group, there was a significant increase in cardiac injury markers like LDH, CK-MB and serum uric acid as compared to vehicle control group which may be due to some extra-cardiac lesion possibly skeletal muscle.

Effect of *CUA* on electrocardiogram:
Heart rate was significantly increased in isoprenaline control group and *CUA* group as compared to vehicle control group. There was significantly decreased R-R interval in *CUA* group as compared to vehicle control group.

Effect of *CUA* on biomarkers of oxidative stress:
In isoprenaline control group, there was significantly decreased in SOD and significant increased in lipid peroxidase and GSH as compared to vehicle control group suggestive of myocardial damage. In *CUA* group, there was significantly decreased in SOD and significantly increased in lipid peroxidase, GSH as compared to vehicle control group. In the active control group, there was significantly increased in catalase activity as compared to isoprenaline control group.

The *CUA* containing phenolic and flavonoids contents may provide defense against oxidative stress. [23] Flavonoids act as good antioxidants because of free radical scavenging activity and protect tissue against free radical mediated lipid peroxidation and also chelates metal ions [24]

In some of the studies, the oral dose of carvedilol was more than 1 mg/kg. [25] So it may be the possible reason for carvedilol not showing any cardioprotective effect except in histopathological parameters in the present study.

Effect of *CUA* on histopathological changes in rats' heart: In vehicle control group, there was normal heart histopathology found. In isoprenaline control group (posterior wall > anterior wall), there was significant necrosis as compared to vehicle control group, High dose *CUA* group and active control group. So high dose *CUA* shows cardioprotection against isoprenaline-induced myocardial damage. The *CUA* group was found as similar as to vehicle control group in all these above parameters (Table 2 & 3) which suggest that rise in LDH, CK-MB and uric acid are due to the extracardiac lesion.

CONCLUSION: High dose *CUA* shows the cardioprotective effect but study with higher doses is necessary.

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