Protective role of Kalpaamruthaa in type II diabetes mellitus-induced cardiovascular disease through the modulation of protease-activated receptor-1

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ABSTRACT

Background: Kalpaamruthaa (KA) is a formulatory herbal preparation has beneficial antioxidant, anti-apoptotic and anti-inflammatory properties against cardiovascular damage (CVD).

Objective: The present study was undertaken to investigate the protective role of KA in type II diabetes mellitus-induced CVD through the modulation of protease-activated receptor-1 (PAR1).

Materials and Methods: CVD was developed in 8 weeks after type II diabetes mellitus induction with high fat diet (2 weeks) and low dose of streptozotocin (2 × 35 mg/kg b.w. i.p. in 24 h interval). CVD-induced rats treated with KA (200 mg/kg b.w. in 0.5 ml of olive oil) orally for 4 weeks.

Results: KA increased the activities of enzymatic antioxidants and the levels of non-enzymatic antioxidants in pancreas of CVD-induced rats. KA effectively reduced the lipid peroxides and carbonyl content in the pancreas of CVD-induced rats. KA reduced cellular damage by ameliorating the activities of marker enzymes in plasma, heart and liver. The protective nature of KA was further evidenced by histological observation in pancreas. Further, KA reduced CVD by decreasing the expression of PAR1 in heart.

Conclusion: This study exhibits the defending role of KA in type II diabetes mellitus-induced CVD through altering PAR1.

Key words: Cardiovascular damage, Kalpaamruthaa, oxidative stress, protease-activated receptor-1, type II diabetes mellitus

INTRODUCTION

Hyperglycemia in diabetes produces multiple biochemical consequence including oxidative stress and plays a vital role in the development of cardiovascular damage (CVD).[1] Chronic hyperglycemia persuades cellular oxidative stress, resulting in the production of oxygen free radicals, a response that plays a major task in the etiology of diabetic complications.[2] Hyperglycemia induces increased superoxide anion production via the activation of multiple pathways including NAD (P) H oxidase, cyclooxygenase, uncoupled nitric oxide synthase, glucose autoxidation, polyol pathway and formation of advanced glycation end products.[3-7] Reactive oxygen species (ROS) can induce cell death via lipid peroxidation (LPO), alteration of cellular proteins and initiation of diverse stress-signaling pathways. Oxidative stress is currently suggested as a major factor underlying the tissue damage in diabetes mellitus and diabetic complications.[8] The increased production of ROS may destruct pancreas and progress β-cell dysfunction in diabetic condition.[9] Protease-activated receptor-1 (PAR1) is a G protein-coupled receptor, is expressed in cardiomyocytes and cardiac fibroblasts and is activated by the coagulation protease thrombin, as well as other proteases. The expression of PAR1 is found to be localized in the endothelial cells of normal arteries, and also it is observed in the smooth muscle cells and macrophages of the vessel wall in atherosclerotic lesions.[10] A common signaling pathway of PAR1 is the activation of phospholipase C, resulting in the formation of inositol triphosphate and diacylglycerol, followed by calcium (Ca$^{2+}$) mobilization and activation of protein kinase C. In our previous study, we have reported that Kalpaamruthaa (KA) modulates CVD by altering protein kinase C/Akt signaling.[11] Hence in this study, we intend to investigate the role of KA on PAR1, a upstream
Kalpaamruthaa is a herbal preparation, consisting of *Semecarpus anacardium* (SA) Linn. nut milk extract, *Emblica officinalis* dried powder of fruit and honey in proposed ratio. SA Linn. nut shells contain biflavones A, C, A1, A2, tetrahydrorobustaflavone B (tetrahydrorobustoflavone), jeediflavone, semecarpuflavone, and galuflavone.[16-19] *Emblica officinalis* has emblicanin A, emblicanin B, punigluconin, pedunculaglin, rutin and gallic acid, which were established by high-performance thin layer chromatography.[20] KA has been reported for its potent antioxidant, anti-inflammatory, anti-arthritis, anticancer and analgesic, antipyretic and non-ulcerogenic properties.[20-24] Further, in our previous study, KA has been reported for its potent antioxidant, anti-apoptotic and anti-inflammatory properties.[25,26] KA effectively improved the endothelial damage, and metabolic alterations in diabetes-induced CVD.[27,28] The goal of this study was to evaluate the protective role of KA in CVD through the modulation of PAR1 expression.

**MATERIALS AND METHODS**

**Chemicals**

Streptozotocin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). SA has been prepared according to Formulary of Siddha Medicine (1972).[29] To this, a fresh dried powder of EO fruit and honey were added. All the other chemicals and reagents used in this study were of analytical grade.

**Animals**

The experiments were conducted according to ethical norms approved by the Ministry of Social justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (Approval No. 01/03/2010). Male *Sprague-Dawley* rats (160–180 g) were obtained from the Central Animal House, Institute of Basic Medical Sciences, Chennai, India. The animals were acclimatized to the laboratory conditions for a period of 2 weeks. The animals were housed under standard conditions of temperature 25°C ± 1°C, relative humidity 60% ± 5%, 12 ± 1 h light/dark cycles. Animals were given a standard rat feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. The efforts were taken carefully to minimize the animal suffering.

**Experimental design**

The animals were divided into five groups with six animals in each group. Group I: Control rats received olive oil (1 ml) during the treatment period. Group II: CVD-induced rats. Insulin resistance was developed by the administration of high-fat diet (84.3% standard laboratory chow; 10% yolk powder, 5% lard, 0.5% bile salt and 0.2% cholesterol) for 2 weeks[30] and then diabetes was induced by streptozotocin administration, 2 × 35 mg/kg body weight intraperitoneally (dissolved in 0.5 ml of 0.1 M citrate buffer, pH 4.5) in 24 h interval, CVD developed in 8 weeks as we reported earlier[25,31] Group III: CVD-induced rats (as group II rats), then treated with KA (200 mg/kg b.w. in 0.5 ml of olive oil) orally for 4 weeks. Group IV: CVD-induced rats (as group II rats), then treated with SA (200 mg/kg b.w. in 0.5 ml of olive oil) orally for 4 weeks. Group V: KA control rats received (200 mg/kg b.w. in 0.5 ml of olive oil) orally during the treatment period.

At the end of the experimental period, animals were sacrificed by decapitation. Blood was collected in a heparinized tube, and plasma was separated by centrifugation at 1000 × g for 10 min. Pancreas, heart and liver were carefully dissected, rinsed in ice-cold physiological saline, homogenized with 0.1 M Tris-HCL buffer (pH 7.4) at 4°C and centrifuged at 800 × g for 10 min at 4°C. The resultant supernatants were used to measure biochemical parameters.

**Biochemical assays**

The activities of enzymatic antioxidants and the levels of non-enzymatic antioxidants were studied in pancreas. Superoxide dismutase (SOD) was measured by the method of Marklund and Marklund.[32] Catalase (CAT) was estimated by Sinha’s method.[33] Glutathione peroxidase (GPx) was assayed according to the method of Rotruck et al.[34] Glutathione reductase (GR) was measured by Staal et al.[35] Reduced glutathione (GSH) was assayed by the method described by Moron et al.[36] Vitamin C was measured by the method of Omaye et al.[37] Vitamin E was assayed according to the method of Desai.[38] LPO was measured by the method of Ohkawa et al. and protein carbonyl levels were also estimated according to the method of Levine et al. in pancreas.[39,40]
Latha, et al.: KA modulates PAR1 during CVD

The levels of LPO and carbonyl in pancreas of control and experimental groups of rats are shown in Figure 1. The levels of LPO and protein carbonyls were increased in CVD-induced rats as compared to control (P < 0.01). KA treatment (P < 0.01) decreased these levels in CVD-induced rats toward near normal than the reduction seen in SA treatment as compared to CVD-induced rats. No difference between control and drug alone was observed.

Table 1 represents the effect of KA on the activities/levels of antioxidants in pancreas of control and experimental groups of rats. The activities of SOD, CAT, GPx and GR, and the levels of GSH, Vitamin C, and Vitamin E were decreased in CVD-induced rats as compared to their control counterparts (P < 0.01). Treated groups (KA and SA) showed a significant increase in their activities. Protection imparted by KA (P < 0.01) was found to be effectively higher than SA treatment as compared to CVD-induced rats. No significant difference was observed between control and KA control groups.

Marker enzymes such as LDH, CK, GOT/AST, GPT/ALT, ALP and γ-GT in plasma, heart and liver of control and experimental groups are summarized in Tables 2 and 3, respectively. CVD-induced rats exhibited increase in these enzyme activities in plasma (P < 0.01) with concordant decrease in heart and liver (P < 0.01) as compared to their control counterparts, whereas treatment with KA and SA ameliorated these activities nearer to normal state. KA imparts better modulatory effect on marker enzymes than the sole SA as compared to CVD-induced rats (P < 0.01). These findings clearly testify the protective effect of KA against CVD.

Figure 2 shows the histological changes of the pancreas in control and experimental groups of rats. Control rats and KA control rats showed intact, big and round clusters of islet cells surrounded by exocrine acini. CVD-induced rats had dilated acini gaps, shrinkage of islet and reduced pancreatic islet area. These pathological findings were altered with normal islet cells, improved islet area and reduced acini gaps in KA and SA treated group of rats. Significant degree modulation was observed in KA treated group.
Table 1: Effect of Kalpaamruthaa on pancreatic antioxidants in control and experimental groups of rats

| Parameters | Control | CVD-induced | Kalpaamruthaa treated | SA treated | Kalpaamruthaa alone |
|------------|---------|-------------|------------------------|------------|---------------------|
| SOD        | 11.73±0.72 | 6.59±0.47**  | 11.00±1.03**          | 8.99±0.75ab | 12.25±1.04         |
| CAT        | 7.47±0.47   | 3.27±0.31**  | 6.01±0.40**           | 4.51±0.35   | 7.48±0.48          |
| GPx        | 12.33±0.92  | 6.57±0.47**  | 11.01±0.85**          | 8.97±0.79   | 12.43±1.00         |
| GR         | 0.89±0.10   | 0.35±0.03**  | 0.81±0.07**           | 0.61±0.06   | 0.91±0.07          |
| GSH        | 3.53±0.32   | 1.84±0.13**  | 3.18±0.21**           | 2.58±0.23   | 3.77±0.23          |
| Vitamin C  | 0.92±0.09   | 0.33±0.03**  | 0.84±0.07**           | 0.53±0.05   | 0.97±0.07          |
| Vitamin E  | 1.10±0.08   | 0.47±0.04**  | 0.93±0.08**           | 0.77±0.05   | 1.14±0.07          |

Values are represented as mean±SD for six rats in each group; values are given statistically significant at *P<0.05; **P<0.01. versus control, versus SA treatment. Enzyme activities are expressed as SOD: Units/min/mg protein (1 unit is equal to the amount of enzyme that inhibits pyrogallol auto-oxidation by 50%); CAT: μmoles of H₂O₂ hydrolyzed/min/mg protein; GPx: μmoles of NADPH oxidized/min/mg protein; GR: μmoles of NADPH oxidized/min/mg protein; GSH, Vitamin C and Vitamin E: μg/mg of protein. SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase; GSH: Glutathione; CVD: Cardiovascular damage; SA: Semecarpus anacardium; SD: Standard deviation

Table 2: Effect of Kalpaamruthaa on marker enzymes in the plasma of control and experimental group of rat

| Parameters | Control | CVD-induced | Kalpaamruthaa treated | SA treated | Kalpaamruthaa alone |
|------------|---------|-------------|------------------------|------------|---------------------|
| LDH        | 82.67±5.95 | 133.25±10.01* | 91.88±7.53**           | 114.90±7.01d | 79.70±6.64         |
| CK         | 172.47±16.54 | 275.51±19.89* | 191.50±15.38**          | 235.88±17.25d | 170.62±11.61       |
| GOT        | 74.60±6.76   | 117.57±7.60** | 79.39±5.02**           | 95.42±8.06** | 73.10±4.87         |
| GPT        | 37.95±2.50   | 58.95±4.32**  | 41.83±3.05**           | 51.20±3.16** | 35.18±2.63         |
| ALP        | 93.39±7.07   | 148.22±11.85** | 100.93±5.59**          | 122.76±9.89d | 91.83±8.29         |
| γ-GT       | 15.60±1.42   | 25.70±2.08**  | 17.37±1.10**           | 21.51±1.53** | 13.82±0.97         |

Values are represented as mean±SD for six rats in each group; Values are considered statistically significant at *P<0.05; **P<0.01. versus control, versus CVD-induced, versus SA treated. Unit: IUL; LDH: Lactate dehydrogenase; CK: Creatine kinase; GOT: Glutamate oxaloacetate transaminase; GPT: Glutamate pyruvate transaminase; ALP: Alkaline phosphatease; γ-GT: γ-Glutamyl transferase; SA: Semecarpus anacardium; SD: Standard deviation; CVD: Cardiovascular damage

Table 3: Effect of Kalpaamruthaa on marker enzymes in heart and liver of control and experimental group of rats

| Parameters | Control | CVD-induced | Kalpaamruthaa treated | SA treated | Kalpaamruthaa alone |
|------------|---------|-------------|------------------------|------------|---------------------|
| Heart      |         |             |                        |            |                     |
| LDH        | 94.95±6.79 | 37.01±3.41** | 80.68±5.53**           | 54.57±4.44ad | 98.49±7.64         |
| CK         | 180.04±15.43 | 66.77±6.21a  | 165.18±13.42**         | 107.14±11.11ad | 189.36±15.92       |
| AST        | 47.07±3.25  | 28.61±2.55** | 42.45±3.46**           | 36.87±2.94** | 50.88±3.39         |
| ALT        | 21.83±1.75  | 11.46±1.11** | 19.87±1.24**           | 16.13±1.41** | 24.71±1.63         |
| ALP        | 190.38±15.55 | 88.04±7.40** | 176.61±12.58**         | 140.02±12.92** | 195.25±12.35       |
| γ-GT       | 12.88±1.01  | 4.47±0.46**  | 10.23±0.90**           | 8.15±0.61**  | 13.08±1.05         |
| Liver      |         |             |                        |            |                     |
| LDH        | 60.43±6.29  | 21.75±1.75** | 55.25±5.14**           | 40.80±3.09** | 62.83±5.76         |
| CK         | 171.91±14.83 | 85.05±6.13a  | 155.34±11.89**         | 115.98±11.85a | 173.68±15.16       |
| AST        | 89.67±6.01  | 37.56±3.13** | 79.35±0.01**           | 55.45±5.54** | 90.29±6.89         |
| ALT        | 27.42±1.69  | 15.90±0.99** | 25.90±1.86**           | 21.04±1.71** | 28.13±1.83         |
| ALP        | 118.16±10.26 | 50.92±3.76** | 106.38±8.99**          | 81.49±5.35** | 122.52±11.33       |
| γ-GT       | 9.51±0.59   | 5.58±0.46**  | 8.97±0.62**            | 7.15±0.61**  | 9.77±0.69          |

Values are represented as mean±SD for six rats in each group; Values are considered statistically significant at *P<0.05; **P<0.01. versus control, versus SA treated. Units: LDH, CK, AST, ALT: N mole of pyruvate liberated/min/mg protein; ALP: N mole of p-nitrophenol released/min/mg protein; γ-GT: N mole of p-nitroaniline released/min/mg protein. LDH: Lactate dehydrogenase; CK: Creatine kinase; GOT: Glutamate oxaloacetate transaminase; GPT: Glutamate pyruvate transaminase; ALP: Alkaline phosphatease; γ-GT: γ-Glutamyl transferase; SA: Semecarpus anacardium; SD: Standard deviation; CVD: Cardiovascular damage

The molecular mechanisms involved in the protective action of KA were studied through PAR1 in heart of control and experimental group of rats. Immunohistochemical expression of PAR1 in myocardium is presented in Figure 3. CVD-induced rats had a high degree of stained PAR1 cells (P < 0.01) as compared to control rats. Treatment with KA reduced the PAR1 expression upon treatment with KA (P < 0.01) as compared with CVD-induced rats. KA administered rats and control rats had minimal number of stained positive cells.
DISCUSSION

Cellular antioxidants are defending against oxidative stress in CVD to protect tissue from damage. This has led to an increase in the demand for natural products with constitutive anti-oxidative property and minimal side effects.\(^{[46,47]}\) In the present study, we found out KA effectively protect against CVD through its antioxidant property and by modulating PAR1 expression.

Oxidative stress in diabetes coexisted with a decline in the antioxidant capacity, which could increase the deleterious effects of free radicals.\(^ {[48]}\) In CVD, the development of oxidative stress is not only by oxygen free-radical generation but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in antioxidants and LPO formation. Increased LPO under diabetic condition can be due to increased oxidative stress in the cell as a result of depletion of antioxidants protective systems.\(^ {[49]}\) CVD-induced rats showed an increase in LPO and protein carbonyl level, which are the evidence of intensified free radical production. These free radicals may react with polyunsaturated fatty acids in the cell membrane leading to peroxidation. KA modulates pancreatic damage with balanced free radicals/antioxidants status by reducing LPO and carbonyl content in our study, suggesting that KA could have a high antioxidant capacity to scavenge free radicals generated by ROS and prevent radical damage. This is in correlation with the previous report that KA reduced the oxidative tissue damage in other experimental condition.\(^ {[23]}\) From the results obtain, KA augmented the enzymatic antioxidants (SOD, CAT, GPs, and GR) and non-enzymatic antioxidants (GSH, Vitamin C and Vitamin E) in the pancreas of CVD-induced condition. Our results are in

**Figure 2:** Histological alterations of pancreas in control and experimental groups of rats. Control rat pancreas (a) had intact, big and round clusters of islet cells surrounded by exocrine acini. Cardiovascular damage-induced (b) rats had dilated acini gaps, shrinked islet and reduced islet area. Kalpaamruthaa treated (c) rats had markedly reduced dilatation of acini and high degree of islet area than Semecarpus anacardium treated (d) rats. Drug alone group of rats (e) had histological pattern similar that of control rats. Sections were visualized under light microscope at a magnification of × 200 (Scale bar - 100 μm)

**Figure 3:** Effect of Kalpaamruthaa (KA) on protease-activated receptor-1 (PAR1) expression in heart of control and experimental group of rats. Immunohistochemical analysis of PAR1 in heart of control and experimental group of rats. (a) Control; (b) cardiovascular damage (CVD)-induced; (c) KA-treated; (d) *Semecarpus anacardium* (SA)-treated; (e) KA alone. Arrows indicate stained positive cells. (f) Quantitative analysis of PAR1 expression. The number of stained (positive) cells per × 200 field was averaged across 15 fields for each rat. Values are given statistically significant at \( P < 0.01 \) denoted by *; \( P < 0.05 \) denoted by #; \( ^{a} \) versus control, \(^{b} \)versus CVD-induced, \(^{c} \)versus SA treated (scale bar - 100 μm)
agreement with the previous report that KA protects tissue from oxidative damage by enhancing antioxidant status.[25]

The marker enzymes namely, LDH, CK, GOT/AST, GPT/ALT, ALP and γ-GT serve as sensitive indices to assess the severity of CVD.[26] When myocardial cells are damaged due to the deficiency of oxygen supply, the cell membrane becomes permeable or may rupture and results in leakage of enzymes ensuing in increased activity of these enzymes in plasma with concomitant decrease in tissue.[31] The activity of CK is an important marker for myocardial damage. LDH is a tetrameric enzyme recognized as a marker with potential use in assessing early stage of myocardial damage in diabetes.[32] γ-Glutamyl transpeptidase has a key task in amino acid transport across membranes and catalyzes the initial step in the breakdown of glutathione. Furthermore, increase in γ-PT activity in plasma is an indicator of impairment in liver function. Measurement of enzymatic activities of aminotransferases (AST and ALT) and ALP is of clinical and toxicological importance as changes in their activities are indicative of tissue damage by toxicants or in disease conditions.[33] The onset of cardiac dysfunction was confirmed with altered activities of these enzymes in CVD-induced rats. Treatment with KA modulated the activities of these enzymes comparable to the control condition. This is an indication of the protective action of KA in reversing cardiac damage. These results are in agreement with the previous report that KA was able to modulate the marker enzymes activities in other experimental system.[34]

The assessment of histological observation of pancreatic section in CVD-induced rats had showed dilated acini gaps, shrinkage of islet and reduction of pancreatic islet area. This result is in agreement with the previous report.[35] KA validates its protective effect by increasing the islet with intact pancreas. This result further substantiates the claim that KA has a protective nature on pancreatic tissue.

Protease-activated receptors have been linked to the regulation of a broad range of cellular functions in cardiovascular systems.[13] PARs are critical for normal homeostasis and contribute to the pathogenesis of vascular disorders characterized by chronic inflammation. Activation of PAR1 mediates responses involved in contractility, inflammation, proliferation and repair.[10] Thus, interference with PAR1 appears to be a promising strategy to treat CVD. It is capable of activating various signaling molecules including protein kinase C and contributes to CVD.[34] KA protects against diabetes-induced CVD by altering protein kinase C/Akt signaling.[11] PAR1 expression was studied in this study to identify the upstream signaling cascade to PKC. In this study, CVD-induced rats had elevated expression of PAR1 in heart. This might be due to increased oxidative stress elicited by hyperglycemia.[12] Treatment with KA reduces the PAR1 expression in CVD-induced rats through its antioxidant and normoglycemic properties.[25,27]

CONCLUSION

This study showed that KA exhibited antioxidant activity, and able to reduce pancreatic oxidative damage. Further, the results of this study suggested that KA is a possible regulator of PAR1 expression and have therapeutic applications in diabetes-induced CVD. These findings provide a significant molecular basis for explaining how KA defends CVD developed in type II diabetes mellitus.

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