AMELIORATIVE POTENTIAL OF ROSUVASTATIN ON DOXORUBICIN-INDUCED CARDIO TOXICITY BY MODULATING OXIDATIVE DAMAGE IN RATS

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INTRODUCTION
Incidence rates of cardiovascular disorders (CVDs) are increasing day by day around the globe particularly developing countries like India, the majority of the mortality rates are due to CVDs despite several advances in medical treatments. Among all CVDs, ischemic heart disease (IHD) like angina pectoris, myocardial infarction (MI) is the most alarming clinical conditions and is the main principle cause of mortality even in developed countries also.1

According to the current status of estimation by 2030, around 23.6 million people will die from only CVDs. Moreover, MI is considered as one of the common forms of IHD leads to irreversible necrosis of cardiac myocytes or myocardial tissue damage due to the failure of vasoregulatory or auto regulatory mechanisms.2

In addition to this, despite advances in the management of CVDs, MI remains the leading cause of mortality around the globe with high incidence rates around the age of 35 years were noticed in male patients which may be due to chronic stress, lack physical activity and lifestyle modifications, etc.3-4 Hence, there is a high demand for research, and innovations were in progress in the field of cardio protection by employing various animal models. In the present study, the DOX-induced cardio toxicity model was used to screen the cardio protective potential in Wistar albino rats.

Doxorubicin is a potent broad-spectrum antibiotic used for the treatment of various cancers. However, the clinical usage has been limited due to its serious side effects such as myocardial injury which is mainly due to mitochondrial dysfunction, apoptosis and the excess generation of free radical leads to cardio toxicity.5-7 Free radical-mediated myocardial damage is an important etiological mechanism that is associated with an increased level of reactive oxygen species and inadequate antioxidant defense system.8,9 Hence, Doxorubicin (DOX) induced MI is the most widely used model to evaluate the cardio protective effect of various drugs, and also the administration of DOX in high doses produces myocardial lesions similar to MI in human.

On the other hand, the test drug of this present study rosuvastatin (ROSS) is a well known inhibitor of the rate-limiting enzyme i.e 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA reductase) for in vivo cholesterol biosynthesis. As per the data, apart from antihyperlipidemic effects, statins possess diverse pharmacological effects including lowering the CVDs and exerts neuroprotective and antioxidant actions.10-13 However, the potential of statins on
cardio protection remains to be explored in different animal models. Therefore, we decided to put some effort to screen the cardio protective effect of ROSS on DOXO induced cardio toxicity in Wistar albino rats.

**MATERIALS AND METHODS**

**Animal care and handling**

Wistar albino rats with a weight range of 160-180 g were used and housed at 25° ± 5°C until the acclimatization period of about one week at Sree Vidyamikethan College of Pharmacy, A. Rangampet, Tirupati. All the animals were maintained under standard experimental conditions according to the guidelines of the Committee for the purpose of control and supervision on experiments on Animals (CPCSEA) and the experimental protocols were duly approved by IAEC. (Institutional Animal Ethics Committee-SVCP/IAEC/I-003/2019-20).

**Drugs, chemicals, and instruments**

Doxorubicin was procured from Sigma-Aldrich, U.S.A whereas assay kits like LDH & CK-MB were supplied by Crest Biosystems, Coral clinical systems, Goa, India. Rosuvastatin was received as a gift sample from Dr.N.N.Palei, Faculty of Pharmacy, Sree Vidyamikethan College of Pharmacy. All biochemical estimation was done using a semi-automatic analyzer (Misma-VIVA, Agappe Diagnostics, Kerala, India) following the methods and stepwise procedures described by the manufacturers.

**Experimental design**

**Morphometric analysis**

Bodyweight of all experimental animals was recorded at regular intervals whereas relative organ weight was calculated post-experimental period after sacrificing the animals. 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.

**Induction of cardio toxicity**

A total of 24 albino rats (150±10gm) was divided by following a random sampling technique into four groups of six animals each (n=6). All the experimental animals were treated as per the study design and the duration of the treatment was continued up to 21 days.

- **Group I** - Rats were given normal saline (1 ml/kg.s.c)
- **Group II** - Rats were given DOXO on 20th day (10 mg/ kg, i.p.)
- **Group III** - Rats were given ROSS (10 mg/kg,p.o)
- **Group IV** - Rats were given ROSS (10 mg/kg,p.o) + DOXO (10 mg/kg,i.p)

Cardiac toxicity was induced by a single intraperitoneal injection of doxorubicin at the dose of 10 mg/ kg on the 20th day of the experiment.14 At the end of the experimental period, After 48 hours of DOXO injection, rats were anesthetized with mild ether anesthesia and blood samples were collected from retro-orbital plexus, and serum was separated by centrifugation at 10000 rpm for 10 min using a centrifuge (REMI, India).

**Estimation of cardiac and liver marker enzymes**

The separated serum sample was used to estimate the creatinine kinase-MB (CK-MB, measured by immune inhibition method by determining the rate of NADPH formation at 340 nm) and liver markers enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), measured by UV kinetic method by observing the rate of nicotinamide adenine dinucleotide reduced form (NADH) formation at 340 nm using an semi-automatic analyzer (Misma-Viva-Agappe Diagnostics) with respective assay kits by following stepwise procedures given by the manufacturer.15-18
Estimation of marker enzymes using post mitochondrial supernatant
Animals were sacrificed and the vital organ like heart was harvested for preparation of post mitochondrial supernatant (PMS). In brief, the harvested heart was per fused with ice-cold normal saline solution and homogenized using a phosphate buffer (0.1 M, pH 7.4) and KCl (1.17%, w/v) solution. Then the homogenate was centrifuged (800 rpm for 5 min at 4°C) to separate the nuclear debris from the mixture. After that, the obtained supernatant was centrifuged at 10500 rpm for 20 min at 4°C to obtain PMS. Collected PMS was used for analyzing for the presence of different enzymes related to myocardial infarction such as creatine kinase-MB fraction (CK-MB) and lactate dehydrogenase (LDH) using an semi-automatic analyzer (Mispa-Viva-Agappe Diagnostics).

Antioxidant activity
Collected post mitochondrial supernatant solution from the heart tissue was used for the measuring superoxide dismutase (SOD) and catalase (CAT) following standard procedures.

Histopathological study
The collected heart tissue was washed with ice-cold normal saline and fixed in formalin (10% neutral) solution followed by embedding in paraffin. Then, it was sectioned (5µm thickness) and stained with hematoxylin and eosin (H.E.) for histopathological examination under a light microscope.

Statistical analysis
The statistical analysis was performed by ANOVA under one-way classification followed by Dunnett's test. p<0.05 was considered statistically significant. Values are expressed as mean ± S.E.M.

RESULTS AND DISCUSSION
Morphometric analysis
Morphometric analysis of all experimental animals is tabulated in Table 1. Results depict that, the body weight of experimental animals was recorded on a weekly interval of drug treatment. From the results, the administration of DOXO had shown significant changes in the bodyweight of animals whereas acute administration of ROSS has shown a remarkable increase (185.39±1.63, p<0.05) in body weight compared to control (171.29±1.36) and DOXO (164.45±2.49) treated group on day 21. On the other hand, Heart weight and relative heart weight of experimental animals are summarized in Table 2. As per the results, heart weight followed by relative heart weight of rats received DOXO were statistically higher than control and ROSS received animals. But, treatment with ROSS significantly (p<0.05) inhibited these weight variation changes influenced by DOXO administration and the obtained results were significantly (p<0.05) comparable with control groups.

Cardiac marker enzymes
The results presented in Figure 1 and Table 3 depict the effect of ROSS on serum CK-MB levels against Doxorubicin-induced cardio toxicity in rats. Results indicate that, doxorubicin received group 2 animals showed a significant raise of CK-MB levels (p<0.05) compared to control animals. However, animals treated in combination with ROSS showed inhibitory action (**p<0.01) on the raising level of CK-MB induced by doxorubicin in rats. Similarly the same trend was noticed in LDH levels too. The results of the effect of ROSS on serum LDH levels were presented in Figure 2 and Table 3. After injection of doxorubicin, we observe that the rats in group II animals showed a marked rise in LDH levels (p<0.05) when compared with control groups. But the results from groups 3 and 4 show the inhibitory action on the toxicity induced by doxorubicin in rats and the results were comparable with control animals.

Post mitochondrial supernatant
The results of the effect of ROSS on heart tissue homogenate CK-MB levels are shown in Figure 3 and Table 3. After the injection of DOXO, we observe that rats in group II animals showed a remarkable rise in CK-MB levels (p<0.05) in tissue homogenate when compared with control groups. But the results from groups III and IV show the inhibitory action (p<0.05) on the toxicity induced by doxorubicin in rats and the results were comparable with control animals.

Liver marker enzymes
The effect of ROSS on serum AST, ALT levels are summarized in Table 4. As per the obtained results, DOXO received animals showed marked rise of both AST (240.34±5.53;***p<0.001) and ALT (103.67±3.44;***p<0.001) whereas acute administration of ROSS significantly reversed these biochemical alterations to a significant extent in case of AST (87.85±4.56; **p<0.01) and ALT (53.72±0.33***; **p<0.01) when compared to DOXO and control animals.

Antioxidant activity
The data depicted in Table 4 & Figure 4,5 reveal that, SOD and CAT levels were reduced in post mitochondrial supernatant solution of heart tissue of DOXO treated group II animals. Administration of DOXO showed marked reduction in SOD (p<0.05) and CAT (p<0.05) at dose level of 10 mg/kg. However, ROSS administration caused a reversal of depleted antioxidants to near normal levels which indicated its protective and antioxidant capabilities.

**Histopathological study**

Histopathological observations of the cardiac tissues of all experimental groups are shown in Figure 6. From the results, cross section of cardiac tissues of control animals showed normal myocardial architecture without any inflammatory cell infiltration whereas necrotic cardiac tissue damage and like proliferated granulation tissues were seen in doxorubicin received animals. However, administration of ROSS reversed these cellular changes to a remarkable significant extent with mild granulation of tissue and restored the normal cellular architecture which reflects its protective potential against cardio toxicity induced by doxorubicin.

**DISCUSSION**

Myocardial infarction is an acute necrotic disorder of the heart and one of the most commonly diagnosed forms of CVD in industrialized nations like India. It is a common and life-threatening manifestation of ischemic heart disease leads to mortality.

Myocardial infarction is the technical name for a heart attack, occurring due to myocardial ischemia resulting from irreversible myocardial tissue necrosis because of complete occlusion of blood vessel (coronary artery) supplying blood to the myocardium. Therefore, the development of cardio protective agents to improve myocardial function is of great clinical importance.

Rosuvastatin has been shown significant organ protective properties with diverse pharmacological effects including anti-inflammatory and antioxidative properties. Furthermore, according to studies, Rosuvastatin reduces oxidative stress by mediating many antioxidant effects, including decreased NADPH oxidase, suppression of endothelial nitric oxide synthase (eNOS) uncoupling, up regulation of antioxidant enzymatic defense mechanisms, and inhibition of hydrogen peroxide-induced DNA harm. But the experimental background found to be fertile or seem to have more gap to support the scientific background of diverse pharmacological profile of ROSS. Hence, the study elucidated the possible \textit{in vivo} ameliorative influence of rosuvastatin in doxorubicin-induced cardio toxicity in rats.

Doxorubicin is a well-known cardio toxic agent capable of inducing cardiac injury in experimental animals and it is widely used model to induce cardio toxicity. Doxorubicin Destroys myocardial cell causing it to release cytosolic enzymes such as CK-MB, LDH, AST and ALT into extra cellular fluid and also in serum which indicates the myocardial tissue damage which was reflected in the study.

Results of the present study indicate that, DOXO caused significant myocardial tissue damage as indicated by a marked raise in the levels of cardiac injury markers, oxidative stress, heart weight, and histological changes in myocardial tissue etc.

The same trend was noticed in heart tissue homogenate of DOX treated animals when compared with the control animals demonstrating the necrotic damage of the myocardial membrane by DOX. Whereas Rosuvastatin treated groups at doses of 10mg/kg alone and in combination demonstrated a significant decline in CK-MB and LDH levels (p<0.05) which ultimately reflects its protective activity against doxorubicin-induced cardio toxicity in rats.

On the other hand, in the pathogenesis of various cardiovascular diseases, reports from earlier studies indicated the involvement of reactive oxygen species and the reduction of antioxidants. In this connection, it is well known that, SOD is one of the important antioxidant enzymes to control many pathological progress in vast disease and disorders.

In this study, increased levels of SOD to a significant extent (p< 0.05), which in turn reflects the protective role of ROSS. The same trend was observed in CAT also. CAT is also considered as one of the vital enzyme made of heme protein and used to scavenge the formed ROS, prevents the tissue damages against free radicals. In addition, histopathological reports further corroborated the obtained results of the present investigations. As per the results, the administration of ROSS reversed cellular changes caused by DOXO to a significant extent and restored the normal cellular architecture which reflects its protective potential against cardio toxicity induced by doxorubicin.

**CONCLUSION**

In conclusion, results from the present study demonstrate that ROSS has shown promising potent cardio protective effects against doxorubicin-induced cardio toxicity in rats as evidenced by the significant reduction of cardiac marker enzymes like CK-MB and LDH both in serum and in heart tissue homogenate along with significant reduction of liver marker enzymes like ALT, AST along with significant beneficial effects were seen in morphometric analysis. On the other hand, it is well recognized that various radical scavengers, such as CAT, SOD,
and GPX, as well as GSH, serve as the first line of defence against oxidative damage, including cardio toxicity. Because reactive oxygen species can cause oxidative damage due to lack of an appropriate endogenous antioxidant defence mechanism. The same were reflected in the present study, which clearly indicated the diminishment of antioxidants such as SOD, CAT in DOXO treated animals. Thus, from the results, oxidative damage was clearly involved in DOXO-induced cardio toxicity. However, the treatment of ROSS remarkably restored DOXO-induced cardio toxicity by up-regulating the antioxidants. Thus, results from invivo antioxidant study reflect that, administration of ROSS significantly raised the level of SOD and CAT along with significant reversal of necrotic tissue damage caused by doxorubicin. Hence it can be concluded that, the cardio protective potential of ROSS may be due to attenuation of cardiac, liver marker enzymes and oxidative stress by modulating oxidative damage by up-regulating the antioxidant defence mechanism.

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Table 1. Effect of ROSS on morphometric analysis on DOXO induced cardio toxicity

| Treatments (mg/kg, b.w) | Body Weight (gr) |
|------------------------|------------------|
|                        | Day 7            | Day 14           | Day 21           |
| CON (1 ml/kg.s.c)      | 143.39±1.67      | 162.58±3.29      | 171.29±1.36      |
| DOXO (10 mg/kg,i.p)    | 154.81±3.78      | 157.92±2.18      | 164.45±2.49      |
| ROSS (10 mg/kg,p.o)    | 152.68±2.62      | 165.37±1.79      | 172.29±1.39      |
| ROSS (10 mg/kg, p.o) + DOXO (10 mg/kg,i.p) | 145.73±2.45  | 173.42±2.56  | 185.39±1.63* |

Values are mean ± SEM, n=5: *p<0.05,**p<0.01,”*p<0.001 When compared to the control group, ns: Non-significant.
Table 2. Effect of ROSS on relative organ weight on DOX-induced cardio toxicity

| Treatments (mg/kg, b.w) | Heart weight (mg) | Absolute Heart Weight | Relative Heart Weight |
|-------------------------|-------------------|-----------------------|----------------------|
| CON (1 ml/kg.s.c)       | 755±3.29          | 0.44±0.06             |
| DOXO (10 mg/kg,i.p)     | 908±2.53          | 0.55±0.08             |
| ROSS (10 mg/kg,p.o)     | 771±1.49          | 0.44±0.04             |
| ROSS (10 mg/kg,p.o) + DOXO (10 mg/kg,i.p) | 774±4.56 | 0.41±0.03 |

Values are mean ± SEM, n=5:

Table 3. Effect of ROSS on Creatine Kinase (CK-MB) and Lactate Dehydrogenase (LDH) levels on DOX-induced cardio toxicity

| Treatments (mg/kg,b.w) | Marker Enzymes | CK-MB (U/L) (Serum) | CK-MB (U/L) (Heart Tissue homogenate) | LDH (IU/L) (Serum) |
|-------------------------|----------------|---------------------|--------------------------------------|--------------------|
| CON (1 ml/kg.s.c)       | 2912.05±4.59   | 222.05±2.47         | 1277±3.47                            |
| DOXO (10 mg/kg,i.p)     | 3157.32±3.62   | 317.32±4.16         | 3560±5.29                            |
| ROSS (10 mg/kg,p.o)     | 1973.37±7.48*  | 299.37±1.49         | 2541±4.58*                           |
| ROSS (10 mg/kg,p.o) + DOXO (10 mg/kg,i.p) | 1091.62±2.36** | 235.62±2.36*        | 2789±4.39*                           |

Values are mean ± SEM, n=5:  *p<0.05, **p<0.01,***p<0.001 When compared to control and toxic control group, ns: Non-significant.

Table 4. Effect of ROSS on liver marker enzymes on DOXO-induced cardio toxicity

| Treatments (mg/kg,b.w) | Liver Marker Enzymes | AST (IU/ml) | ALT (IU/ml) |
|-------------------------|----------------------|-------------|-------------|
| CON (1 ml/kg.s.c)       | 64.23±3.45           | 43.48±2.57  |
| DOXO (10 mg/kg,i.p)     | 240.34±5.53***       | 103.67±3.44*** |
| ROSS (10 mg/kg,p.o)     | 131.67±3.49*         | 73.43±1.29* |
| ROSS (10 mg/kg,p.o) + DOXO (10 mg/kg,i.p) | 87.85±4.56**        | 53.72±0.33** |

Values are mean ± SEM, n=5:  *p<0.05, **p<0.01,***p<0.001 When compared to control and toxic control group, ns: Non-significant.
Table 5. Effect of ROSS on Antioxidant levels on DOX-induced cardio toxicity

| Treatments (mg/kg,b.w)          | Antioxidant Enzymes |
|---------------------------------|---------------------|
|                                 | SOD (µg/mg of protein) | CAT (U/mg of protein) |
| CON (1 ml/kg.s.c)               | 45.86±1.49          | 1.37±0.69             |
| DOXO (10 mg/kg,i.p)             | 32.6±2.32           | 0.77±0.54             |
| ROSS (10 mg/kg,p.o)             | 58.7±1.56*          | 1.41±0.48*            |
| ROSS (10 mg/kg,p.o) + DOXO (10 mg/kg,i.p) | 60.9±1.58* | 1.67±0.53*            |

Values are mean ± SEM, n=5: *p<0.05,**p<0.01,***p<0.001 When compared to control and toxic control group, ns: Non-significant.

Figure 1. Effect of ROSS on serum CK-MB levels on DOX-induced cardio toxicity in rats
Figure 2. Effect of ROSS on serum LDH levels on DOX-induced cardio toxicity in rats

Figure 3. Effect of ROSS on CK-MB in heart tissues homogenate on DOX-induced cardio toxicity in rats
Figure 4. Effect of ROSS on SOD levels on DOXO-induced cardio toxicity
Figure 5. Effect of ROSS on CAT levels on DOXO-induced cardiotoxicity.
Figure 6. Histopathological observations of different experimental animals using hematoxylin and Eosin staining (CON-Control animals showed normal myocardial cellular architecture; DOX-Doxorubicin received groups showed noticeable necrotic cellular tissue damage; ROSS-Rosuvastatin treated groups; DOX + ROSS-Doxorubicin plus rosvastatin treated groups showed significant reversal of necrotic tissue damage caused by doxorubicin.)