Chapter 3: Evaluation of rosmarinic acid against myocardial infarction in maternally separated rats

3.1 Introduction

Childhood abuse, isolation, neglect, and maternal separation (MS) are significant factors that chronically leads to depression [334, 335]. In this context, MS is a considerable risk factor that leads to depression *via* hyperactivation of the HPA axis and oxidative stress [336, 337]. Neglectful parenting is a major worldwide issue for humans [338, 339]. The foremost reasons for neglecting a child or MS are employed women, abandonment, death of the mother, divorce, mother in prison, and boarding schools [340, 341]. MS is characterized by the loss of a mother in childhood, causing depression and cardiovascular diseases due to adverse effects on the HPA axis later in life [342-344].

A recent study reported MS-induced alterations in behavioral, cardiac, and hippocampal functions [345]. In general, depression is manifested by anorexia, insomnia, anhedonia, depressed mood, and loss of interest in activities [346]. The global prevalence of depression is increasing rapidly [347] and is expected as a predominant cause of mortality worldwide by 2030 [348]. The alarming increase in depression cases has been considered a key reason behind the surge in cardiovascular events due to overlapping physiological mechanisms [349, 350].

Myocardial infarction (MI), a most important cause of coronary heart disease, is pathologically characterized by myocardial tissue death due to ischemia [351, 352]. Oxygen derived free radicals are the main cause of ischemia-induced cardiac damage [353]. Oxidative stress destroys the lipid membrane of myocytes leading to myocardial necrosis [354]. A supramaximal dose of isoproterenol elevates the levels of cytotoxic free radicals and lipid peroxidation causing irreversible necrotic damage of cardiac myocytes membrane and fibrosis of cardiac tissue [355, 356].

The severity of MI among depressed patients is higher than any other comorbid diseases such as diabetes, arthritis, and cancer [250, 357]. Moreover, a great body of literature reported that comorbid depression increases the chance of a future coronary event by 30%, which indicates a strong interrelationship in depression and cardiac abnormalities [216, 358]. A recent study reported that depressed patients increased 25 % risk of rising MI [359]. Accumulating evidence suggests that depression and coronary heart disease share several common pathophysiological mechanisms. The common mechanisms have been characterized as hyperactivation of the HPA axis, inflammation, and oxidative stress. Hyperactivation of the HPA axis causes activation of corticosterone, catecholamines, proinflammatory cytokines, and platelet aggregation leading to myocardial tissue death and heart failure [360, 361]. Oxidative stress causes alterations in the endogenous antioxidative system (glutathione and superoxide dismutase) leads to an overproduction of ROS/RNS, a significant cause of myocardial tissue damage in MI [362, 363].

There is an unmet need to develop therapeutic strategies that abrogate the common pathophysiological mechanisms of MI and depression. In our previous study (chapter 2), rosmarinic acid ameliorated the CUS-induced cardiac abnormalities possibly through modulation of serotonergic, oxidative, and inflammatory pathway. The pleiotropic pharmacological activities of RA make it a suitable candidate for managing MI comorbid depression, which involves a myriad of pathophysiological characteristics. However, till date, no studies have been conducted to assess the therapeutic effect of RA against MI in comorbid depression-induced by MS in rats. In the present study, MS has been used to model comorbid depression in rats subjected to MI. Considering all the pathophysiological factors, it can be hypothesized that administration of RA abrogates inflammation, oxidative stress, abnormal cardiac

biomarkers creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH), and imbalance in neurotransmitter levels. In the current study, we explored the potential benefits of RA treatment against MI in comorbid depressed rats, focusing primarily on a clinical situation where depression (maternal stress) affects the severity of MI. Therefore, the current investigation was designed to examine the effect of RA against MI comorbid depression induced by maternal separation through measuring immobility period (forced swim test), anhedonia symptom (sucrose preference test), stress marker (corticosterone), and neurotrophic factor (BDNF). We have measured the effect of RA on oxidative stress (glutathione and superoxide dismutase) and anti-inflammatory cytokine (IL-10) biomarkers to test its efficacy in treating MI comorbid depression. ST-segment alteration (electrocardiography) and cardiac biomarkers (CK-MB and LDH) were quantified to assess the link between maternal stress and MI.

3.2 Materials and methods

3.2.1 Animals and Housing

All rats were procured from the central animal house of the Banaras Hindu University, Varanasi, India, and housed in a standard vivarium condition (25 ± 2 °C and 12-h light/12-hr dark cycle). Food and water are available *ad libitum*. A total of 48 pups were tested up to 8 weeks. Institutional Animal Ethics Committee approved all the protocols, Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology, Banaras Hindu University, Varanasi, India IIT(BHU)/IAEC/2022/055, and experiments were performed according to Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) guidelines.

3.2.2 Maternal Separations

The male and female rats (1:2) were housed together and allowed to mate for one week. After birth, pups were subjected to MS as described previously [364, 365]**.** Briefly, 70−80% of pups were separated from their dam daily for 3 h between 9:00 AM to 12:00 PM from postnatal day-1 (PND1) up to postnatal day-21 (PND21) and kept in a thermoregulated chamber at 32°C in the separated room to prevent communication *via* ultrasonic vocalization [366]. At PND21, all pups were weaned, and siblings were separated by gender and housed in a particular group. The delivery day was considered as PND0. The remaining pups were left undisturbed in their respective home cages throughout the preweaning period and considered non-maternal separation/non-handled (N-MS/NH) subjects.

3.2.3 Chemicals

Rosmarinic acid was procured from Sigma Aldrich, India. Fluoxetine was procured from Umang Pharmacy, Institute of Medical Science, Banaras Hindu University. Isoproterenol (ISO) hydrochloride (100 mg/kg) was procured from Sigma Aldrich and dissolved in normal saline to get a 100 mg/mL solution. The dosage of RA, fluoxetine, and isoproterenol was selected based on the previous experimental studies [240, 241, 367, 368]. Aqueous suspensions of RA and fluoxetine were prepared using 0.5% tween-80. The aqueous suspensions of RA (25 mg/kg and 50 mg/kg) and fluoxetine (10 mg/kg) was administered through oral gavage.

3.2.4 Induction of experimental myocardial infarction

In this study, two consecutive subcutaneous injections of isoproterenol (100 mg/kg/body weight) were used to induce myocardial infarction on PND53 and PND54 at an interval of 24 h [239, 369].

3.2.5 Experimental design

After the birth, pups were randomly allocated into eight different groups. Group I consisted of naïve pups (non-maternal separated group), Group II consisted of maternal separated pups (MS group), Group III consisted of control group received ISO 100 mg/kg, Group IV consisted of MS group received ISO 100 mg/kg, Group V control group received RA (50 mg/kg), Group VI consisted of MS group received ISO (100 mg/kg) and RA (25 mg/kg), Group VII consisted of MS group received ISO (100 mg/kg) and RA (50 mg/kg), and Group VIII consisted of MS group received ISO (100 mg/kg) and fluoxetine (10 mg/kg). The drugs (rosmarinic acid and fluoxetine) were administered to groups VI, VII, and VIII starting from PND35 to PND55. The inclusion of group-V (RA 50 mg/kg alone) was to ascertain the impact of rosmarinic acid on normal pharmacological parameters in the absence of stressor and isoproterenol. In general, any drug that affects normal pharmacological parameters may lead to potential side effects. Fluoxetine was used as a model validator considering its clinical use against post-MI depression and anxiety [370, 371]. The research design is demonstrated in *Figure 17.*

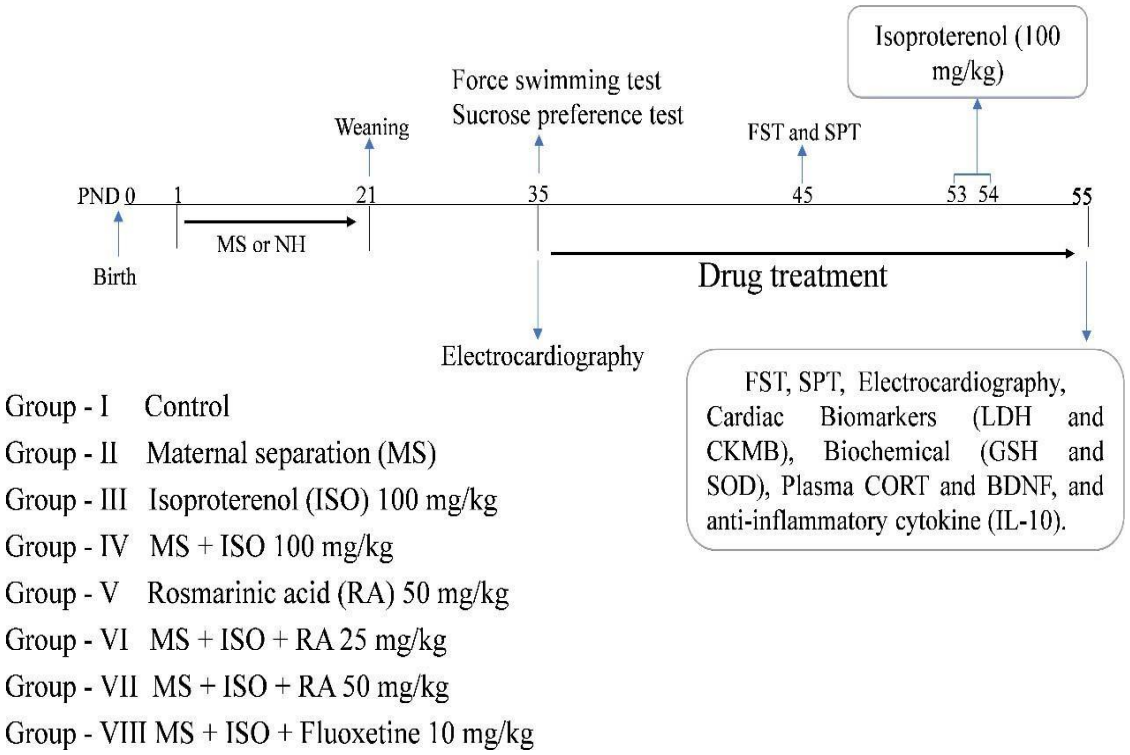

Figure 17: Schematic representation of experimental design.

RA; Rosmarinic Acid, PND; Postnatal Days, MS; Maternal Separation; NH; Non-Handled, MS; Maternal Separation, ISO; Isoproterenol, FST; Forced Swim Test, SPT; Sucrose Preference Test, CK-MB; Creatin Kinase-MB, LDH; Lactate Dehydrogenase, GSH; Glutathione, CORT; Corticosterone, BDNF; Brain Derived Neurotrophic Factor, IL-10; Interleukin-10.

3.2.6 Body weight

Body weight was measured throughout the experiment and body weight of each pup on PND0 were compared with the body weights of PND35 and PND55.

3.2.7 Electrocardiography

Electrocardiography was performed on PND35 (after induction of MS) and PND55 (24 h after second isoproterenol injection) under isoflurane anaesthesia (2% in 100% oxygen at 300 mL/min following induction in a chamber, containing 3-5% isoflurane) [245, 246]. Electrocardiograms were recorded using LabScribe software through the iWire-B3G ECG module, IXTA data acquisition unit, electrode lead wires,

and three ECG electrodes (red, black, and green) per rat. Body temperature was maintained between 37–38 °C using a heating pad.

3.2.8 Forced swim test

A forced swim test is the most widely and well-established core model of depressive-like behavior in rodents [372]. In this model, the duration of immobility is estimated to measure depressive-like behavior in MS rats. On PND34 of the experiment, each rat was kept in a plexiglass cylinder for 15 min during swimming training. After the training period, rats were removed from the cylinder and dried adequately before returning to their home cages. On the PND35, rats were allowed to swim for 5 min and the immobility period was recorded. Similarly, the immobility periods were recorded on PND45 and PND55. The experimental conditions were maintained as described previously [251, 252]

3.2.9 Sucrose preference test

In the sucrose preference test, anhedonia is a behavioral sign of depressive-like behavior evaluated as decreased sucrose consumption by rats [255, 373]. Briefly, after adaptation with 1% w/v sucrose solution for two days, on the PND35, PND45, and PND55 of the experiment, each rat was kept in a single isolated cage for 1 h and allowed free access to two separate bottles containing a pre-measured quantity of 1% w/v sucrose solution and tap water. After the drinking session, the content of the bottles is re-measured and recorded to estimate sucrose and water consumption. The percent sucrose preference was calculated as described previously [16, 255].

$$\text{Sucrose preference (\%)} = \frac{\text{sucrose consumption (mL)}}{[\text{sucrose consumption (mL)} + \text{ water consumption (mL)}]} \times 100$$

3.2.10 Collection of blood, tissues, and homogenate preparation

On the last day of the experiment, rats were anesthetized under CO_2, and blood was collected in two different heparinized and non-heparinized tubes through a retro-orbital puncture. Plasma and serum were separated from the blood by centrifugation at 1500 g and 3000 g, respectively, for 10 min at 4 °C and stored at −80 °C for estimation of CK-MB, LDH, and IL-10 level. After sacrificed, the brain and heart were removed, weighed, and homogenized in cold phosphate-buffered saline (0.1M, pH 7.4) to prepare 10% tissue homogenate. The tissue homogenate was centrifuged at 10,000 × g for 20 min at 4 °C, and the supernatant was collected and stored at −80 °C until assayed.

3.2.11 Estimation of biochemical parameters

According to the manufacturer's instructions, the plasma corticosterone (Abcam, USA), BDNF (My BioSource Inc., USA), and IL-10 (Ray Biotech Inc., GA, USA) were measured using enzyme linked immunosorbent assay kit. All the reagents were brought at room temperature before use, and the assay was performed according to protocol instructions, and absorbances were recorded using iMark microplate reader at 450 nm. In addition, the severity of the myocardial injury was assessed through quantification of cardiac biomarkers CK-MB and LDH in plasma using enzymatic kit (Span Diagnostic, India) according to the manufacturer's instructions. The plasma corticosterone levels (ng/mL), BDNF levels (pg/mL), IL-10 levels (pg/mL), and cardiac biomarkers (CK-MB and LDH in IU/L) were calculated from the standard curve.

3.2.12 Estimation of level of antioxidative enzymes

In tissue homogenate, glutathione (GSH) content and superoxide dismutase (SOD) activity were quantified as oxidative stress markers. The GSH content was estimated in brain and heart tissue homogenate by the Ellman assay method [374].

Briefly, 20 µL of the sample or standard solution, 75 µL Tri-HCL pH 8.2, 25 µL dithionitric benzoic acid (DTNB), 25 µL methanol were added to an Eppendorf tube and centrifuged at 3,000 x g for 5 min at room temperature. The mixture was incubated at 37 °C for 10 min, and absorbances were recorded at 412 nm using an iMark microplate reader (Bio-Rad Laboratory, USA). The concentration of GSH was calculated from the standard curve and value expressed in nmol/mg of protein [368]. According to the manufacturer's instructions, the SOD activity was measured using a commercially available SOD kit (Sigma, USA) [250]. The SOD activity was calculated from a standard curve, and values were expressed in unit/mg of protein.

3.2.13 Statistical analysis

In this experiment, quantitative data were represented as the mean ± standard deviation (SD), and the inferential statistics were performed using GraphPad Prism version 7.03 for Windows (GraphPad Software Inc., USA). The data sets with one independent variable were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The difference between the two means was considered statistically significant if the p value was $p < 0.05$.

3.3 Results

3.3.1 Effect of rosmarinic acid on behavioral test

3.3.1.1 Effect of rosmarinic acid on forced swim test and sucrose preference test

The effect of rosmarinic acid on duration of immobility in the forced-swim test, anhedonia behavior in sucrose preference test, and body weight was measured to evaluate the depressive-like behavior on PND35, PND45, and PND55. In figure 18A and 18B, significant ($p < 0.0001$) changes in the immobility periods and percentage of sucrose preference were observed in MS-control compared to control group on PND35 and PND45. Similarly, on PND55, a significant ($p < 0.0001$) increase in the immobility period and decrease in the sucrose preference were observed in MS-control and isoproterenol group compared to control group. However, MS combined with isoproterenol significantly ($p < 0.0001$) increased the immobility period and decreased the sucrose preference as compared to MS-control group on PND55. Interestingly, administration of rosmarinic acid 25 mg/kg significantly ($p < 0.001$) decreased the immobility period but insignificant ($p > 0.05$) increased the sucrose preference compared to MS-control group. However, MS treated rosmarinic acid 50 mg/kg significantly ($p < 0.0001$) decreased the immobility period and increased the sucrose preference as compared to MS-control group. Administration of fluoxetine 10 mg/kg significantly ($p < 0.0001$) decreased the immobility period and increased the sucrose preference compared to MS-control and MS treated rosmarinic acid 25 mg/kg group on PND35 and PND45. However, on PND55, a significant ($p < 0.0001$) decrease in immobility period and increase in sucrose preference were observed in rosmarinic acid 25 mg/kg and 50 mg/kg group compare to MS combined isoproterenol group. In contrast, fluoxetine at 10 mg/kg significantly reduced the immobility period compared to MS combined isoproterenol ($p < 0.0001$) and rosmarinic acid 25 mg/kg ($p < 0.01$)

group and increased the sucrose preference compared to MS combined isoproterenol ($p < 0.0001$) and rosmarinic acid 25 mg/kg ($p < 0.0001$) group on PND55. As expected, only rosmarinic acid 50 mg/kg treatment did not show any significant change in immobility period and percentage of sucrose preference compared to control group (figure 18A and 18B) *Figure 18*.

3.3.1.2 Effect of rosmarinic acid on body weight

We next determined the effect of rosmarinic acid on body weight in experimental rats (figure 18C). It was found that the body weight was significantly increased in MS-control group (123.3 ± 8.7 g, $p < 0.01$, figure 18C) compared to control group (100.0 ± 14.1 g, $p < 0.01$, Figure 18C) on PND35. Similarly, on PND55, body weight was significantly increased in MS-control (166.6 ± 7.5 g, $p < 0.0001$, figure 18C) while non-significantly increased in isoproterenol group (151.8 ± 8.7 g, $p > 0.05$, figure 18C) compared to control group (143.3 ± 8.1 g, figure 18C). Similarly, MS combined isoproterenol group (164.6 ± 5.1 g, $p > 0.05$, Figure 18C) showed insignificant effect compared to MS-control and isoproterenol group. Treatment with rosmarinic acid at a dose 25 mg/kg (108.3 ± 10.3 g, $p > 0.05$, figure 18C) and 50 mg/kg (103.3 ± 15.05 g, $p > 0.05$, figure 18C) insignificantly decreased the body weight as compare to MS-control group at PND35. Similarly, on PND55, treatment with rosmarinic acid at a 25 mg/kg (158.3 ± 4.9 g, $p > 0.05$, figure 18C) and 50 mg/kg (153.3 ± 5.3 g, $p > 0.05$, figure 18C) insignificantly decreased the body weight compared to MS combined isoproterenol group. In contrast, fluoxetine 10 mg/kg significantly decreased the body weight on PND35 [(93.3 ± 12.1 g, $p < 0.001$, figure 18C) and PND55 (146.6 ± 9.8 g, $p < 0.001$, figure 18C)] compared to MS-control group, respectively. However, treatment with rosmarinic acid 50 mg/kg did not show any significant changes in body weight compared to control group (*Figure 18*).

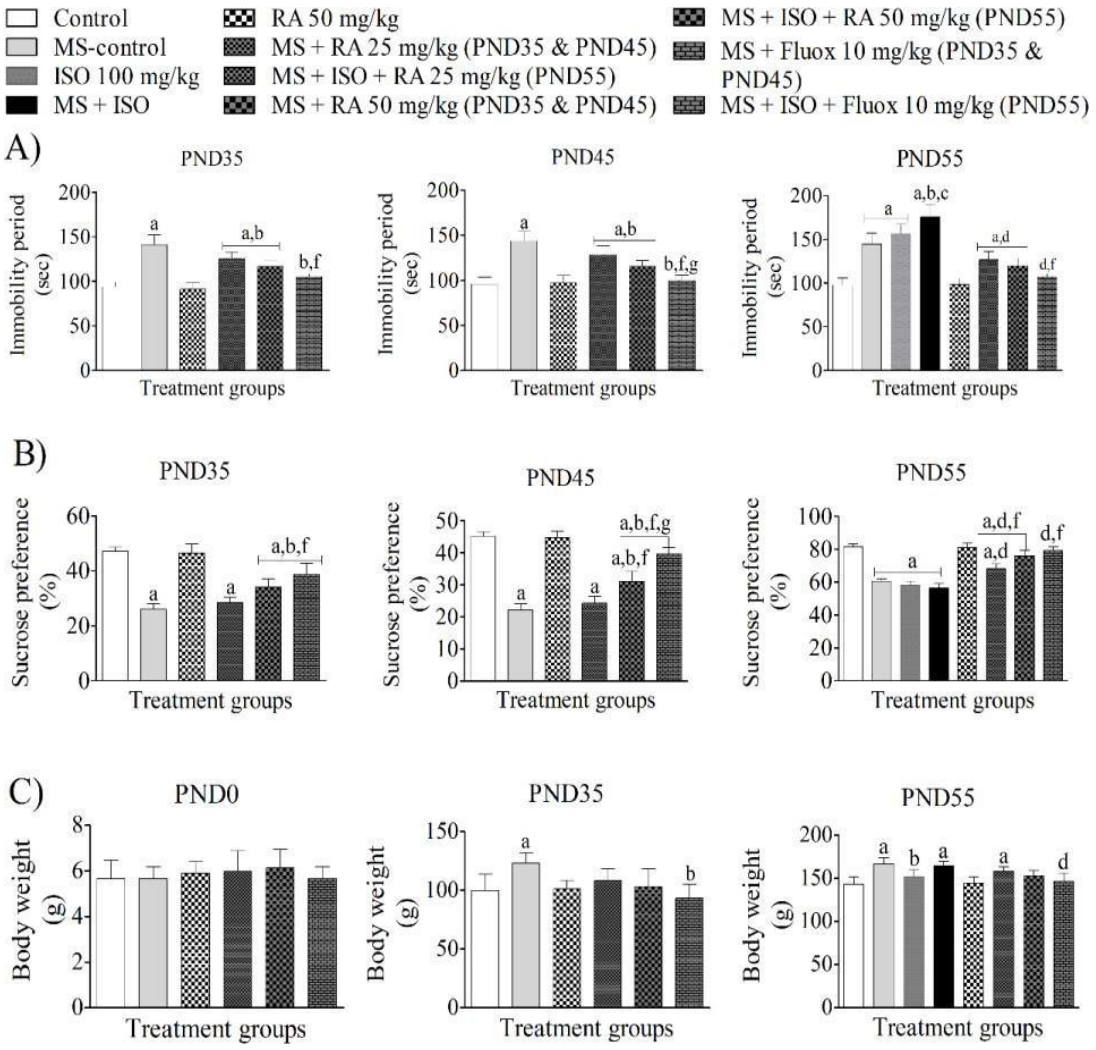

Figure 18: Treatment with rosmarinic acid and fluoxetine decreased depressive-like behavior.

The absolute values for PND35, PND45, and PND55 are shown in parenthesis after the symbols. For immobility period (A): $^{a}p < 0.0001$ vs Control [(93.8 ± 5.03); (95.6 ± 7.84); (97.66 ± 8.09) sec], $^{b}p < 0.0001$ vs MS-control [(141.0 ± 11.09); (143.5 ± 10.38); (144.5 ± 12.70) sec], $^{c}p < 0.0001$ vs ISO 100 mg/kg (156.5 ± 10.78 sec, only for PND55), $^{d}p < 0.0001$ vs MS + ISO (175.6 ± 14.15 sec, only for PND55), $^{e}p < 0.0001$ vs RA 50 mg/kg [(90.83 ± 7.30); (97.0 ± 8.31); (98.5 ± 6.62) sec], $^{f}p < 0.0001$ vs MS + RA 25 mg/kg [PND35, (125.3 ± 7.31); PND45, (128.3 ± 9.85) sec], $^{f}p < 0.0001$ vs MS + ISO + RA 25 mg/kg (126.5 ± 9.69 sec, for PND55), $^{g}p < 0.0001$ vs MS + RA 50 mg/kg [PND35, (116.6 ± 6.86); PND45, (115.3 ± 6.62) sec], $^{g}p < 0.0001$ vs MS + ISO + RA 50 mg/kg (119.8 ± 8.58 sec, for PND55), $^{h}p < 0.0001$ vs MS + fluoxetine 10 mg/kg [PND35, (105.0 ± 3.09); PND45, (100.0 ± 5.47) sec] and $^{h}p < 0.0001$ vs MS + ISO + fluoxetine 10 mg/kg (107.0 ± 3.40 sec, for PND55). For sucrose preference (B): $^{a}p < 0.0001$ vs Control [(47.1 ± 1.4); (45.1 ± 1.4); (81.5 ± 1.7) %], $^{b}p < 0.0001$ vs MS-control [(26.0 ± 2.1);

(22.1 ± 2.04); (60.6 ± 1.5) %], [c]p < 0.0001 vs ISO 100 mg/kg (58.3 ± 2.3 %, only for PND55), [d]p < 0.0001 vs MS + ISO (56.5 ± 2.5 %, only for PND55), [e]p > 0.05 vs RA 50 mg/kg [(46.5 ± 3.27); (44.6 ± 2.06); (81.1 ± 2.48) %], [f]p < 0.0001 vs MS + RA 25 mg/kg [PND35, (28.5 ± 1.8); PND45, (24.3 ± 1.06) %] and [f]p < 0.0001 vs MS + ISO + RA 25 mg/kg (68.3 ± 2.9 %, for PND55), [g]p < 0.0001 vs MS + RA 50 mg/kg [PND35, (34.1 ± 3.06); PND45, (31.0 ± 3.3) %] and [g]p < 0.0001 vs MS + ISO + RA 50 mg/kg (76.1 ± 3.3 %, for PND55), [h]p < 0.0001 vs MS + fluoxetine 10 mg/kg [PND35, (38.6 ± 4.03); PND45, (39.6 ± 1.9) %], [h]p < 0.0001 vs MS + ISO + fluoxetine 10 mg/kg (79.1 ± 2.5 %, for PND55). For body weight (C): [a]p < 0.0001 vs Control [(PND35, p < 0.01, 100.0 ± 14.1, g) and (PND55, p < 0.0001, 143.3 ± 8.1, g)], [b]p < 0.01 vs MS-control [(PND35, p < 0.01, 123.3 ± 8.7, g) and (PND55, p < 0.0001, 166.6 ± 7.5, g)], [c]p > 0.05 vs ISO 100 mg/kg (151.8 ± 8.7 g, only for PND55), [d]p < 0.0001 vs MS + ISO (164.6 ± 5.1 g, only for PND55), [e]p > 0.05 vs RA 50 mg/kg [PND35, (101.1 ± 7.65, g) and PND55, (144.83 ± 7.05, g)], [f]p > 0.05 vs MS + RA 25 mg/kg (108.3 ± 10.3 g, for PND35), [f]p > 0.05 vs MS + ISO + RA 25 mg/kg (158.3 ± 4.9 g, for PND45), [g]p > 0.05 vs MS + RA 50 mg/kg (103.3 ± 15.05 g, for PND35) and [g]p > 0.05 vs MS + ISO + RA 50 mg/kg (153.3 ± 5.3 g, for PND55), [h]p < 0.001 vs MS + fluoxetine 10 mg/kg (93.3 ± 12.1 g, for PND35) and [h]p < 0.001 vs MS + ISO + fluoxetine 10 mg/kg (146.6 ± 9.8 g, for PND55). [One-way ANOVA followed by Tukey's test]. All values are represented as Mean ± SD (n = 6).

3.3.2 Effect of rosmarinic acid on biochemical parameters

3.3.2.1 Effect of rosmarinic acid on plasma corticosterone

In order to evaluate the depressive-like behavior, we determined the levels of plasma corticosterone, a well validated marker of stress in rodents. We found that, MS-control (55.4 ± 5.37 ng/mL, p < 0.0001, figure 19A) and isoproterenol (56.5 ± 5.32 ng/mL, p < 0.0001, figure 19A) independently caused a significant elevation in plasma corticosterone level compared to the control group (31.8 ± 0.7 ng/mL, p < 0.0001, figure 19A). Similarly, MS-isoproterenol combination group (64.7 ± 1.3 ng/mL, p < 0.01, figure 19A) further increased the production of plasma corticosterone levels compared to the MS-control and isoproterenol group. Interestingly, rosmarinic acid at dose 25 mg/kg (49.3 ± 5.2 ng/mL, p < 0.0001, figure 19A) and 50 mg/kg (40.1 ± 4.4

ng/mL, $p < 0.0001$, figure 19A) showed significant and dose-dependent decrease in plasma corticosterone compared to MS combined isoproterenol group. However, rosmarinic acid 50 mg/kg was more effective than rosmarinic acid 25 mg/kg. Similarly, fluoxetine 10 mg/kg exhibited significant reduction in plasma corticosterone level (33.04 ± 3.5 ng/mL, $p < 0.0001$, figure 19A) compared to MS combined isoproterenol and rosmarinic acid 25 mg/kg group. The administration of rosmarinic acid 50 mg/kg alone did not affect plasma corticosterone levels compared to the control group (*Figure 19*).

3.3.2.2 Effect of rosmarinic acid on BDNF and IL-10 levels

BDNF and altered circulating cytokine (IL-10) profile are associated with depressive disorders and coronary heart events [216, 375, 376]. In maternal stress and MI, a suppressed expression of these markers has been reported in several pre-clinical and clinical studies [377-379]. A similar trend was observed in the present study. Post hoc analysis revealed a significant decline in levels of BDNF [(8.19 ± 1.37 pg/mL, $p < 0.0001$, figure 19B and 6.92 ± 1.19 pg/mL, $p < 0.0001$, figure 19B)] and IL-10 [(40.5 ± 4.86 pg/mL, $p < 0.0001$, figure 19C and 33.7 ± 2.70 pg/mL, $p < 0.0001$, figure 19C)] in MS-control and isoproterenol group compared to the control group [(11.45 ± 1.08 pg/mL, $p < 0.0001$, figure 19A) and (61.59 ± 2.39 pg/mL, $p < 0.0001$, figure 19C)]. However, MS combined with isoproterenol further caused substantial reduction in levels of BDNF (5.02 ± 0.91 pg/mL, $p < 0.01$, figure 19B) and IL-10 (26.30 ± 3.76 pg/mL, $p < 0.0001$, figure 19C) compare to the MS-control and isoproterenol group as depicted in *Figure 19*. Interestingly, RA 25 mg/kg [(8.5 ± 0.38 pg/mL, $p < 0.0001$, figure 19B) and (49.02 ± 1.77 pg/mL, $p < 0.0001$, figure 19C)] and RA 50 mg/kg [(10.01 ± 0.33 pg/mL, $p < 0.0001$, figure 19B) and (56.45 ± 4.30 pg/mL, $p < 0.0001$, figure 19C)] caused significant increase in levels of BDNF and IL-10 compared to MS

combined isoproterenol group. However, fluoxetine at 10 mg/kg significantly increased the BDNF (11.0 ± 0.66 pg/mL, $p < 0.0001$, figure 19B) and IL-10 (60.97 ± 1.03 pg/mL, $p < 0.0001$, figure 19C) levels compared to MS combined isoproterenol and RA 25 mg/kg group. As expected, treatment with rosmarinic acid 50 mg/kg alone did not show any significant change in BDNF (figure 19B) and IL-10 (figure 19C) levels compared to the control group (*Figure 19*).

3.3.2.3 Effect of rosmarinic acid on plasma CKMB and LDH levels

CK-MB and LDH are important assessment cardiac biomarkers with cardiotoxicity linked to MI abundantly present on myocardial tissue. In this study, to investigate the severity of cardiac injury, we estimated plasma CK-MB and LDH levels in all treatment groups. The results revealed that MS-control and isoproterenol group caused significantly increased in CKMB [(511.66 ± 40.70 IU/L, $p < 0.0001$, figure 19D and 551.66 ± 25.77 IU/L, $p < 0.0001$, figure 19D)] and LDH [(605.0 ± 44.15 IU/L, $p < 0.0001$, figure 19E) and (650.83 ± 23.75 IU/L, $p < 0.0001$, figure 19E)] levels as compared to control group [(246.66 ± 24.22 IU/L, $p < 0.0001$, figure 19D) and (198.33 ± 40.70 IU/L, $p < 0.0001$, figure 19E)]. However, MS combined isoproterenol group showed substantial increase in CK-MB (639.83 ± 94.53 IU/L, $p < 0.01$, figure 19D) and LDH (780.0 ± 41.47 IU/L, $p < 0.0001$, figure 19E) levels compared to MS-control and isoproterenol independent group. Interestingly, MS combined isoproterenol group increased CK-MB and LDH levels, indicating an increase in the severity of further cardiac damage due to comorbid MS-induced depression. Remarkably treatments with RA at dose of 25 mg/kg and 50 mg/kg demonstrated significant effects in CKMB (331.66 ± 31.88 IU/L, $p < 0.0001$, figure 19D and 273.33 ± 49.66 IU/L, $p < 0.0001$, figure 19D) and LDH (383.33 ± 61.53 IU/L, $p < 0.0001$; figure 19E and 271.66 ± 66.75 IU/L, $p < 0.0001$, figure 19E) levels compared to MS combined isoproterenol.

However, MS combined isoproterenol treated RA 50 mg/kg showed significant ($p < 0.001$) effect as compared to MS combined isoproterenol treated RA 25 mg/kg group. Similarly, fluoxetine 10 mg/kg significantly decreased the level of CKMB (255.0 ± 49.29 IU/L, $p < 0.0001$, figure 19D) and LDH (258.33 ± 46.22 IU/L, $p < 0.0001$, figure 19E) in maternally separated rats indicating reduction in the severity of cardiac damage. As expected, treatment with rosmarinic acid 50 mg/kg alone did not show any significant change in CKMB (figure 18D) and LDH (figure 18E) levels compared to the control group (*Figure 19*).

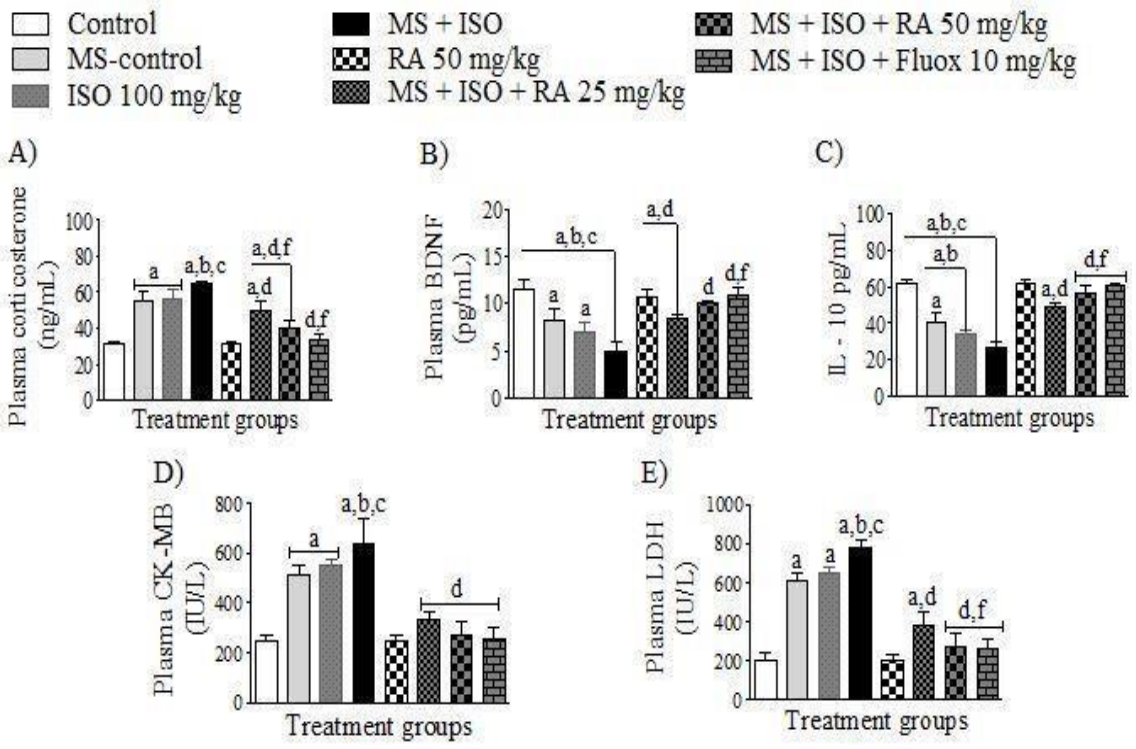

Figure 19: Rosmarinic acid and fluoxetine treatment attenuated the increased plasma corticosterone, CKMB and LDH levels and decreased BDNF and IL-10.

[a]$p < 0.0001$ versus Control, [b]$p < 0.0001$ versus MS-control, [c]$p < 0.0001$ versus ISO 100 mg/kg, [d]$p < 0.0001$ versus MS + ISO, [e]$p < 0.0001$ versus RA 50 mg/kg, [f]$p < 0.0001$ versus MS + ISO + RA 25 mg/kg, [g]$p < 0.0001$ versus MS + ISO + RA 50 mg/kg, [h]$p < 0.0001$ vs MS + ISO + fluoxetine 10 mg/kg [One-way ANOVA followed by Tukey's test]. All values are represented as Mean ± SD (n = 6).

3.3.3 Effect of rosmarinic acid on oxidative stress parameters

Glutathione is a major free radical scavenger provides protection against chronic stress and cardiac damage. In this study, one-way ANOVA showed a significant difference in brain and heart tissue GSH levels. Our results revealed that the MS-control group have decreased the GSH level [(brain; 0.259 ± 0.087 versus 0.647 ± 0.089, $p<0.0001$ and heart; 0.346 ± 0.094 versus 0.674 ± 0.068, nmol/mg of protein, $p < 0.0001$, figure 20A)] and SOD activity [(brain; 9.44 ± 0.84 versus 17.03 ± 1.06 and heart; 4.88 ± 0.83 versus 8.56 ± 0.18, Unit/mg of protein, $p < 0.0001$, figure 20B)] compared to control group. Similarly, isoproterenol group decreased the level of GSH [(brain; 0.166 ± 0.025 versus 0.647 ± 0.089, $p<0.0001$ and heart; 0.188 ± 0.024 versus 0.674 ± 0.068, nmol/mg of protein, $p < 0.0001$, figure 20A)] and SOD activity [(6.56 ± 0.18 versus 0.647 ± 0.089 and heart; 4.05 ± 0.11 versus 8.56 ± 0.18 Unit/mg of protein $p < 0.0001$, figure 20B)] compared to the control group. Further, MS combined isoproterenol caused a considerable reduction of GSH level [(brain; 0.042 ± 0.020 and heart; 0.048 ± 0.011 nmol/mg of protein, $p<0.01$, figure 20A)] and SOD activity [(brain; 4.47 ± 1.09, $p < 0.0001$ and heart; 2.55 ± 0.70 versus 4.88 ± 0.83, $p < 0.0001$, Unit/mg of protein, figure 20B)] compared to the MS-control and isoproterenol independent group. In MS combined isoproterenol group, decreased GSH level and SOD activity in heart representing increased severity of myocardial damage due to comorbid MS-induced depression (*Figure 20*). However, treatment with RA at dose 25 mg/kg significantly increased the level of GSH [(brain, 0.296 ± 0.024, $p < 0.0001$ and heart, 0.484 ± 0.073, nmol/mg of protein, $p < 0.0001$, figure 20A)] and SOD activity [(brain, 11.09 ± 1.32 and heart, 5.66 ± 0.91, $p > 0.05$, figure 20B)] compared to MS combined isoproterenol group. Similarly, RA 50 mg/kg substantially increased the levels of GSH [(brain, 0.550 ± 0.092, $p < 0.0001$ and heart, 0.548 ± 0.106 nmol/mg of

protein, $p < 0.0001$, figure 20A)] and SOD activity [(brain, 13.79 ± 1.14 $p < 0.0001$, and heart, 6.60 ± 0.25 Unit/mg of protein, $p < 0.0001$, figure 20B) compare to the MS combined isoproterenol group rats. In contrast, fluoxetine 10 mg/kg significantly increased the GSH level [(brain, 0.590 ± 0.096, $p < 0.0001$ and heart, 0.615 ± 0.050 nmol/mg of protein, $p < 0.0001$, figure 20A)] and SOD activity [(brain; 15.60 ± 0.45, $p < 0.0001$ and heart; 7.37 ± 0.70 Unit/mg of protein, $p < 0.0001$, figure 20B)] in maternally separated rats. However, rosmarinic acid 50 mg/kg alone did not affect GSH levels (figure 20A) and SOD activity (figure 20B) compared to control group.

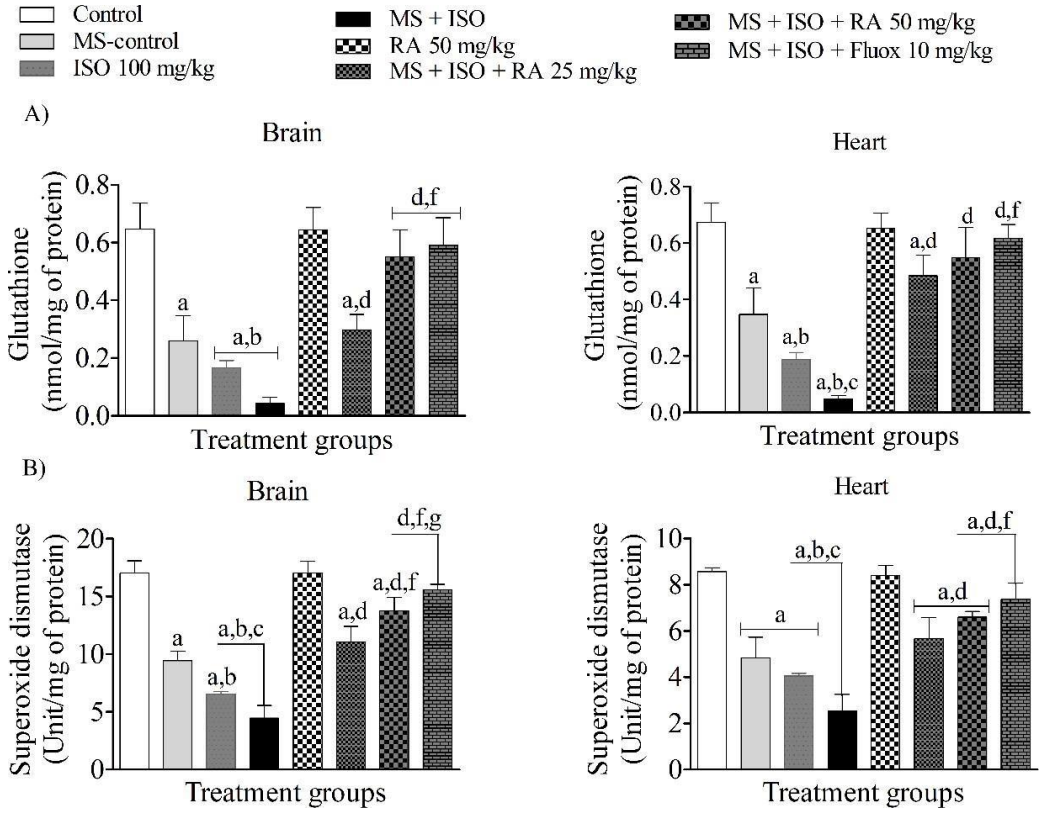

Figure 20: Rosmarinic acid and fluoxetine treatment increased GSH level and SOD activity in brain and heart of maternally separated rats.

[a]$p < 0.0001$ versus Control, [b]$p < 0.0001$ versus MS, [c]$p < 0.0001$ versus ISO 100 mg/kg, [d]$p < 0.0001$ versus MS + ISO, [e]$p < 0.0001$ versus RA 50 mg/kg, [f]$p < 0.0001$ versus MS + ISO + RA 25 mg/kg, [g]$p < 0.0001$ versus MS + ISO + RA 50 mg/kg, [h]$p < 0.0001$ vs MS + ISO + fluoxetine 10 mg/kg [One-way ANOVA followed by Tukey's test]. All values are represented as Mean ± SD (n = 6).

3.3.4 Effect of rosmarinic acid on ST-segment elevation

Electrocardiography (ECG) is a predominant diagnostic technique for cardiac abnormalities, used as a diagnostic pointer, and gives mechanistic information. We used the ECG method to identify MI and to investigate MS, isoproterenol, and MS combined isoproterenol-induced changes in the ST-segment. Representative ECG tracings of rats from each group showed in *Figure 21*. During exposure to MS, isoproterenol, and MS combined isoproterenol, a significant difference in ST-segment (mV) was observed among groups on PND35 and PND55. In our study, MS alone caused a significant ($p < 0.0001$) increase in ST-elevation compared to the control group on PND35. However, on PND55, isoproterenol significantly ($p < 0.0001$) increased the ST-elevation compared to the control group. Similarly, MS combined isoproterenol caused substantial significantly ($p < 0.0001$) increase in ST-elevation compare to MS-control and isoproterenol group at PND55. MS combined isoproterenol extensively increased the ST-elevation compared to isoproterenol alone, indicating increased severity of myocardial damage due to comorbid MS-induced depression. Intrestingly, rosmarinic acid at dose 25 mg/kg and 50 mg/kg significantly ($p < 0.0001$) decreased ST-elevation compared to MS-control (PND35 and PND55) and MS combined isoproterenol rats (PND55). However, rosmarinic acid 50 mg/kg showed more extensive effect as compared to rosmarinic 25 mg/kg group rats. In contrast, fluoxetine 10 mg/kg showed significant effect compared to MS-control ($p < 0.0001$), RA 25 mg/kg ($p < 0.0001$) and RA 50 mg/kg ($p < 0.01$) on PND35. Similarly, on PND55, fluoxetine treatment showed significant ($p < 0.0001$) effect as compared to MS-control and RA 25 mg/kg group and reinstated the ST-segment in maternally separated rats. As observed with other parameters, no significant change in ST-segment was observed in rosmarinic acid 50 mg/kg group compared to control group (*Figure 22*).

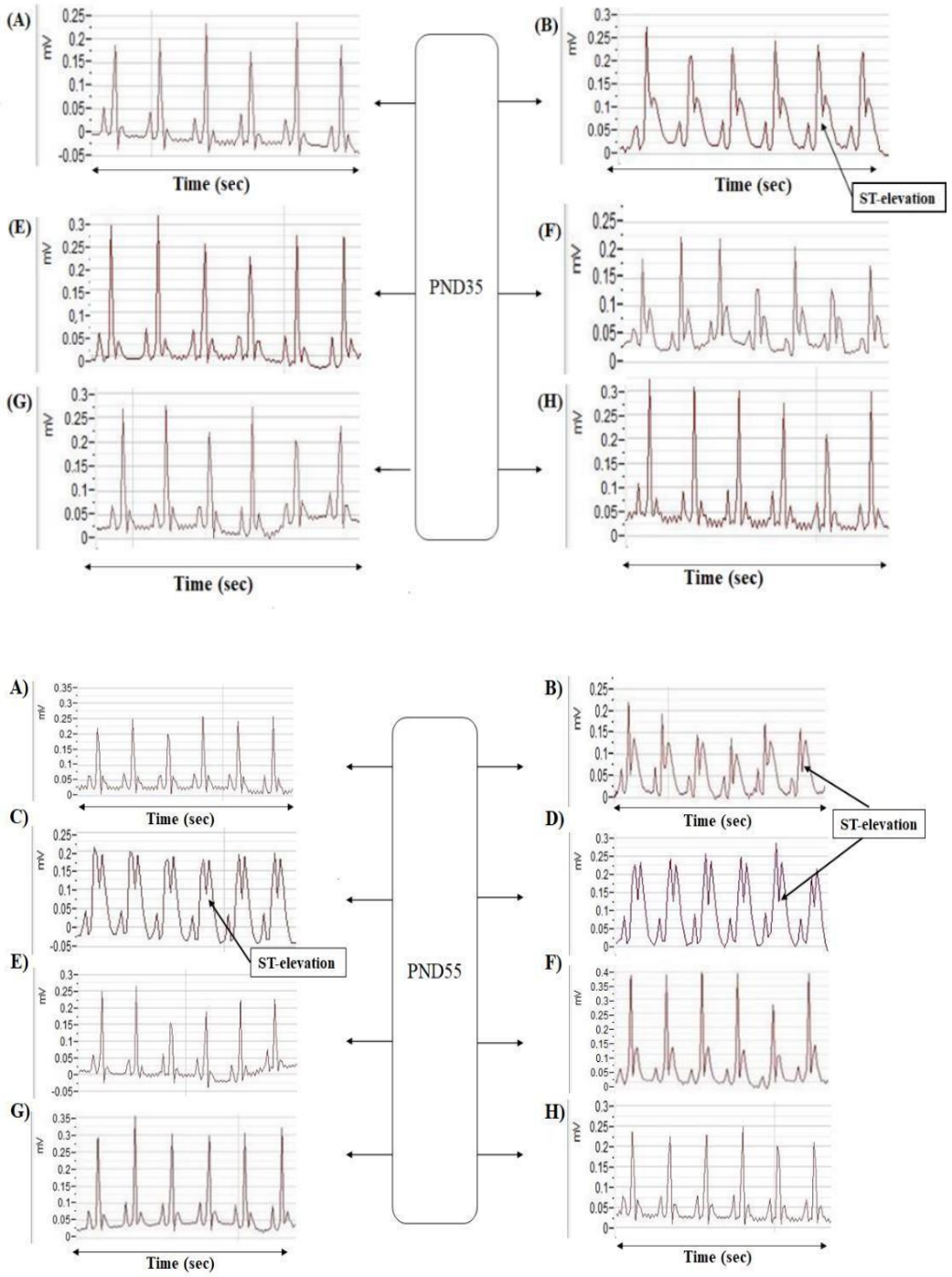

Figure 21: Effects of rosmarinic acid and fluoxetine treatment on electrocardiographic pattern at PND35 and PND55.

A) Control, B) MS-control, C) ISO 100 mg/kg, D) MS + ISO, E) RA 50 mg/kg, F) MS + ISO + RA 25 mg/kg, G) MS + ISO 100 mg/kg + RA 50 mg/kg, H) MS + ISO + fluoxetine 10 mg/kg.

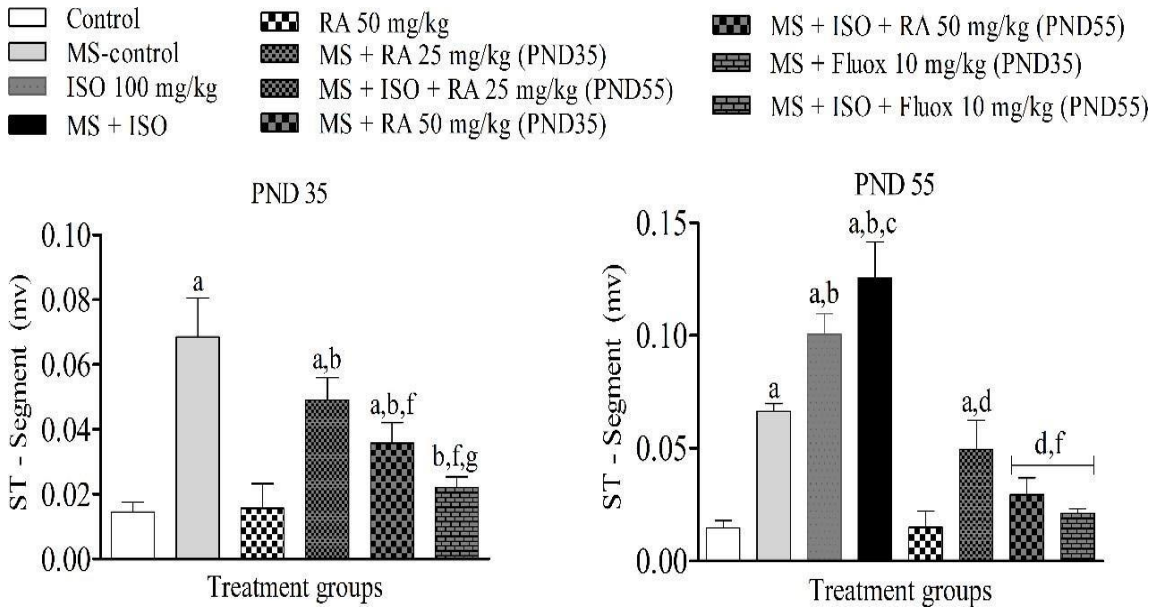

Figure 22: Rosmarinic acid and fluoxetine treatment reduced the ST-elevation at PND35 and PND55.

The absolute values for PND35 and PND55 are shown in parenthesis after the symbols. $^{a}p < 0.0001$ vs Control [(0.0145 ± 0.003); (0.0146 ± 0.0032) mV], $^{b}p < 0.0001$ vs MS-control [(0.068 ± 0.01); (0.066 ± 0.003) mV], $^{c}p < 0.0001$ vs ISO 100 mg/kg (0.100 ± 0.009 mV, only for PND55), $^{d}p < 0.0001$ vs MS + ISO (0.125 ± 0.01 mV, only for PND55), $^{e}p < 0.0001$ vs RA 50 mg/kg [(0.015 ± 0.0075); (0.014 ± 0.007) mV], $^{f}p < 0.0001$ vs MS + RA 25 mg/kg (0.049 ± 0.0069 mV, for PND35), $^{f}p < 0.0001$ vs MS + ISO + RA 25 mg/kg (0.0495 ± 0.013 mV, for PND55), $^{g}p < 0.0001$ vs MS + RA 50 mg/kg (0.035 ± 0.0064 mV, for PND35), $^{g}p < 0.0001$ vs MS + ISO + RA 50 mg/kg (0.029 ± 0.007 mV, for PND55), $^{h}p < 0.0001$ vs MS + fluoxetine 10 mg/kg (0.022 ± 0.003 mV, for PND35), $^{h}p < 0.0001$ vs MS + ISO + fluoxetine 10 mg/kg (0.021 ± 0.002 mV, for PND55). [One-way ANOVA followed by Tukey's test]. All values are represented as Mean ± SD (n = 6).

3.4 Discussion

Due to the unsafe side effects of marketed medicines, there has been rising attention to potential nutritional therapies for amelioration of neuropsychiatric and cardiovascular diseases [380, 381]. The present study evaluated naturally derived polyphenol rosmarinic acid as potential therapeutic option against MI in comorbid depressed rats.

Our finding revealed that MS and isoproterenol independently increased the immobility period, body weight, corticosterone, ST-segment, cardiac biomarkers (CK-MB and LDH) while decreasing the level of sucrose consumption, BDNF, IL-10, glutathione, and superoxide dismutase activity. Remarkably, MS combined isoproterenol further exacerbated the all-aforementioned abnormalities and increased the severity of myocardial infarction in comorbid depression induced by maternal separation. Moreover, the administration of rosmarinic acid and reference drug - fluoxetine significantly ameliorated all of the complications mentioned above caused by MS, isoproterenol, and MS combined isoproterenol. Rosmarinic acid diminished the MS-induced myocardial infarction, may be because rosmarinic acid possesses widely anti-oxidant, anti-inflammatory, antidepressant, and BDNF modulatory properties. Hence, these outcomes might present a novel discovery that may guide clinical therapies against MI in comorbid depressed individuals.

The ISO induced MI in rodents is a simple non-surgical model with postsurgical complications and mortality rate [382, 383]. The supramaximal dose of isoproterenol in this model demonstrates morphological aberrations in rat's myocardium similar to those observed in human hence suitable for screening of natural as well as synthetic compounds [384, 385]. The consequences of this animal study stated that

administration of rosmarinic acid and reference drug may considerably decrease ST-elevation, cardiac biomarkers such as CKMB and LDH to confirm it protective action against myocardial infarction induced by MS and isoproterenol in rats. Isoproterenol induce MI in rats mainly *via* oxidative stress, coronary artery occlusion, cardiac hyperactivity, coronary hypotension, and ischemia [310, 386]. However, future studies using more relevant animal models of myocardial infarction are warranted to generalize the effect rosmarinic acid against myocardial infarction induced by maternal separation.

In humans and rodents, adverse events experienced early in postnatal life have been correlated with an augmented risk of brain and heart associated dysfunctions during the latter part of the life, which further persists throughout the life leading to psychiatric disorders and cardiac abnormalities [345, 387]. Researchers reported that early life stress, including MS, has been proven as a robust predictor of cardiovascular diseases and ischemic heart disease [388, 389].

Maternal separation is an efficient model widely used to study the adverse effects of early life stress on brain function and structure linked to depression [17, 390]. Considerable evidence showed that the MS model is successful when pups were separated from their mothers for up to two to three weeks for 3 h period [391, 392]. MS model is liable to expand depressive-like symptoms due to stress induced hyperactivation of the HPA-axis and imbalance in neurotransmitter levels [393-395]. Dysfunction of the HPA-axis in chronic stress is a major mechanism contribute in depression [396-398]. In addition, due to hyperactivation of HPA-axis in early life chronic stress leading to an augmentation of corticosterone level leading to cascade of endocrine events [399, 400]. Furthermore, supply of neurotrophic factor such as BDNF, alleviates the neurotoxic insults effect of corticosterone [401]. Moreover, isoproterenol

shows a stimulatory effect on HPA-axis *via* pituitary/hypothalamic β-receptors causing augmentation of corticosterone production [402]. In the present study, MS, isoproterenol, and MS combined isoproterenol exacerbated the production of corticosterone levels. In addition, we found rosmarinic acid decreased the corticosterone level in a similar way of reference treatment (fluoxetine), bupropion, and amitriptyline [293]. Consequently, we demonstrated that similar to fluoxetine, rosmarinic acid at 50 mg/kg showed significant attenuation of corticosterone level than dose 25 mg/kg possibly due to higher BDNF modulatory properties. This result strongly suggests that rosmarinic acid and fluoxetine showed protective effect in maternally separated rats by regulating plasma corticosterone levels and normalize the hyperactivity of HPA-axis.

Moreover, BDNF is an endogenous neurotrophic protein, strongly implicated in antidepressant activity *via* expression of corticotropin-releasing hormone and HPA-axis modulation [403]. In addition, studies showed that BDNF level is an important biomarker and abundantly present in plasma and brain [404, 405]. BDNF involves maintaining the integrity of the vascular system in adults *via* cardiac afferent fibres and neural signals protecting ischemic heart injuries [406]. Previous studies stated that MS for 21 days causes a reduction of BDNF level due to dysfunction in neuronal plasticity in the later part of the life of rats [407, 408]. However, after MI, BDNF expressions are increased in the brain but not in the heart due to ablation of afferent nerves from the heart, which decreases BDNF level leading to cardiac dysfunction [406]. Nonetheless, BDNF expressions is upregulated by neural signals from the heart, and this circulating BDNF protects from ischemic injuries signifying a brain-mediated mechanism through which BDNF showed cardiac protective action [406]. Cardiac afferent nerve fibres convey chemo sensitive and mechanosensitive signals to the brain, and these signals

regulate the cardiac function in MI [409, 410]. In our study, BDNF level was decreased in MS-control, isoproterenol, and MS combined isoproterenol group. In contrast, rosmarinic acid increased the plasma BDNF levels in maternally separated rats with an insignificantly higher increase with rosmarinic acid 50 mg/kg, suggesting modulation of BDNF may require higher doses of rosmarinic acid. Besides our results, some recent studies found that rosmarinic acid promotes BDNF level and neurogenesis in the hippocampus subjected to stress [411, 412]. BDNF is an essential neurotrophic factor that favors the serotonergic system and regulates body behaviors [413, 414]. Support to these mechanisms, in MS, BDNF level is reduced, leading to negatively influences on body behavior observed by increased immobility period and decreased sucrose preference in stressed subjects [415, 416]. Similarly, in our study, MS, isoproterenol, and MS combined isoproterenol increased the immobility period and anhedonia behavior. However, fluoxetine treatment significantly attenuated the immobility period and anhedonia behavior as compared to rosmarinic acid (25 and 50 mg/kg) treatment possibly due to higher BDNF modulatory property of fluoxetine than the tested doses of rosmarinic acid.

Moreover, considerable evidence suggests that body weight gain is predominant in maternally separated subjects [396]. In a previous study, MS pups showed hyperphagia and weight gain after weaning, possibly due to the impact on psycho-emotional behavior, social isolation, and decreased metabolic rate [417, 418]. Similarly, in our study, the maternally separated animals showed body weight gain, whereas animals that received only isoproterenol did not show any change in body weight. However, at PND35 and PND55, rosmarinic acid 50 mg/kg and fluoxetine significantly reduced the body weight compared to rosmarinic acid 25 mg/kg possibly by improving

psycho-emotional behavior, BDNF modulatory action, and balance in the serotonergic system, which normalized the diet and body weight in maternally separated rats.

Furthermore, inflammation is a major factor which is associated with increased expression of adhesion molecules and infiltrations of neutrophils and macrophages in many diseases. These activated leukocytes lead to increase in pro-inflammatory cytokines and decrease in anti-proinflammatory cytokines [419, 420]. Anti-inflammatory cytokine (IL-10) is another biomarker that suppresses the overproduction of steroids and contributes to the regulation of HPA-axis homeostasis [421]. In MI and depression, chronic stress may play a critical role in immune activation mediated through oxidative stress [422, 423]. A decreased production of specific anti-inflammatory cytokines such as IL-10 has been linked to MI and depression [424, 425]. In contrast, several studies show that cytokines also affect the adrenal system in the HPA-axis [426]. Besides that, IL-10 is a central operating cytokine that regulates the HPA-axis by suppressing ACTH hormone-induced corticosterone production [427]. IL-10 regulates the innate immune system by inhibiting inflammatory host response and proinflammatory cytokines systems [427]. In depressed patients, low levels of IL-10 are not sufficient enough to counterbalance the hyperactivation of proinflammatory cytokines and hypersecretion of corticosteroids [421, 428]. Consequently, fluctuations in anti-inflammatory cytokines (IL-10) are associated with clinical depression and predisposition to depressive symptoms and cardiac diseases [429, 430]. Along with depressive illness, IL-10 protects the myocardium from ischemic injury or MI [424, 431]. In our finding, MS combined isoproterenol group showed decreased level of IL-10 indicating increased severity of cardiac damage in comorbid depressed rats. Similar to fluoxetine, rosmarinic acid increased the levels of IL-10 possibly due to higher anti-

inflammatory activities, which provides protective effect against MI in comorbid depressed rats.

Electrocardiography is considered the single competent initial clinical test for detecting MI (life-threatening factor), characterized by ST-segment elevation [309]. Along with it, several other symptoms involve such as tachycardia and QRS voltage reduction [432]. Our study found that MS combined isoproterenol substantially increased the elevation of the ST-segment, indicating increased severity of MI, possibly due to the loss of plasma membrane in the damaged myocardial tissue membrane because of stress [364, 433]. Remarkably, we found that rosmarinic acid successfully reduced the ST-elevation in maternally separated rats in a dose-dependent manner. It suggests that rosmarinic acid protects against the severity of MI in comorbid depression induced by MS. This protection could be related to improved myocardial tissue health conferred by the antioxidative and anti-inflammatory effects of rosmarinic acid. Oxidative stress and inflammation are two predominant factors involved in damaging the membrane of myocardial tissue [434, 435]. The significant rise in cardiac enzymes (CK-MB and LDH) due to excessive activation of the peripheral sympathetic system followed by emotional stress [436, 437]. These enzymes are classic indexes of heart tissue damage or myocardial injury, which is well proved in previous studies [438, 439]. In the present study, rats exposed to either MS or isoproterenol showed a significant increase in plasma cardiac biomarkers (CK-MB and LDH) indicating myocardial tissue damage. Because of cardiomyocytes oxidative stress, cardiovascular protein leak in to plasma fluid. Moreover, MS combined isoproterenol group rats showed considerable increase in plasma CK-MB and LDH levels indicating increased severity of MI in comorbid depression. An earlier study reported that rosmarinic acid extract (for 3 weeks) significantly prevents alterations in the permeability of the

myocardial membrane and decreases the level of CK-MB and LDH in streptozocin induced diabetic rats [310, 440]. Similarly, in our study, administration of rosmarinic acid reversed the increased levels of CK-MB and LDH possibly by preventing alteration in permeability or integrity of myocytes membrane/protective membrane or antioxidative property.

There are pieces of evidence that an imbalance between the oxidative and antioxidative systems play an essential role in the pathogenesis of various diseases such as depressive disorders, MI, and heart failure [441, 442]. Hence, the quick reduction of oxidative stress is essential for maintaining the health of the brain and heart tissue because oxidative stress may cause necrotic lesions in the brain and heart tissue [443, 444]. Reactive oxygen species and free radicals are the main factors for oxidative stress *via* GSH and SOD depletion [445, 446]. These GSH and SOD work as anti-oxidants that play an essential role in detoxifying ROS/RNS and protecting tissue damage [447]. The vital part of defending free radicals consists of antioxidative enzymes such as GSH, SOD, and catalase to maintain tissue health [448]. Due to the higher scavenging of free radicals, phenolic compounds are usually used for maintaining the level of antioxidative systems in the body [449, 450]. Therefore, we evaluated the protective role of rosmarinic acid against MS-induced MI in comorbid depression, in which oxidative stress plays a mechanistic role, and measured GSH and SOD activity in brain and heart tissue. The brain and heart tissue are more susceptible to ROS/RNS due to lower anti-oxidant enzymes and natural metabolic activities [451, 452]. The previous study reported that MS-induced oxidative stress leads to cell damage by decreased anti-oxidants levels in tissues [453].

Rosmarinic acid may exhibit a protective effect towards MS-induced oxidative damage because it has remarkable anti-oxidant properties [454-456]. In the present study, MS, isoproterenol, and MS combined with isoproterenol caused oxidative stress, which was identified by reduced GSH levels and SOD activity in the brain and heart tissue. In addition to GSH, SOD enzymes are also involved as anti-oxidants and work *via* depletion of free radicals (O_2^{--} and OH^-) and conversion of superoxide radicals into H_2O_2 and oxygen [448, 457]. Researchers reported that the level of SOD activity decreases in depressive disorders and MI [246, 458]. Similar to previous studies [459, 460], present study showed that rosmarinic acid and fluoxetine treatment amended the decreased levels of GSH and SOD through anti-oxidant property.

Fluoxetine administration resulted in reduction of all the parameters of depression and the downstream markers of cardiac damage significantly better than rosmarinic acid. Considering the fact that depression induces cell damaging inflammatory and oxidative pathways, our results depict that reduction in depression-like behavior was the major reason behind the cardioprotective effects.

1.9 Conclusion

The results of our present study showed that MS independently caused ST-elevation and increased cardiac biomarkers (CK-MB and LDH), which are significant features of MI. Furthermore, MS exacerbated the severity of MI induced by isoproterenol, indicating MS as a predisposing factor in the severity of myocardial tissue damage. Rosmarinic acid (25 and 50 mg/kg) and fluoxetine (10 mg/kg) treatment showed a potent cardioprotective effect by increasing the levels of BDNF, IL-10, GSH, and SOD activity in maternally separated rats. Moreover, rosmarinic acid reversed the ST-elevation and cardiac biomarkers (CK-MB and LDH) levels in maternally separated

rats. We further found that rosmarinic acid treatment diminished excessive corticosterone levels, immobility period, and anhedonia symptoms. Consequently, rosmarinic acid provides a novel therapeutic option for cardio protection against myocardial infarction in comorbid depressed rats *via* anti-inflammatory, antioxidative, anti-depressive, and BDNF modulatory properties.