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Fasudil, a Rho kinase inhibitor, attenuates cardiovascular changes in an experimental rat model of metabolic syndrome via modulation of PCSK9 and BNP

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Abstract:

Introduction: Metabolic syndrome (MS) is an important cardiovascular risk factor. Rho-kinase (ROCK) is a novel cardiovascular therapeutic target and its activity is increased in patients with MS.

Aim of the study: To investigate the impact of Fasudil, a Rho kinase inhibitor, on the Rho-ROCK pathway, nitric oxide (NO), brain natriuretic peptide (BNP) and cardiac PCSK9/LDL concentrations, and to explore its potential role in improving insulin resistance and cardiovascular abnormalities.

Materials and Methods: 24 adults male Wistar albino rats were used and divided into 4 groups: Control, Fasudil1 (10 mg/kg/day subcutaneously), Sucrose-fed (30% in drinking water), Sucrose + Fasudil group. daily for 3 weeks. Blood pressure and heart rate, serum biomarkers (ROCK1, BNP, NO, insulin, glucose and LDL-c), and cardiac PCSK-9 concentrations were assessed in addition to cardiac histopathology.

Results: The data proved that sucrose + Fasudil treatment significantly ameliorated the levels of systolic blood pressure, glycemic indices, serum LDL, Rho kinase, NO, BNP and cardiac PCSK9 compared to the untreated sucrose group. Histology examination confirmed the positive impact of Fasudil on cardiac inflammation, fibrosis and tissue degeneration compared to the untreated.

Conclusion: Fasudil has a cardioprotective effect with improved glycemic indices on sucrose-induced MS in rats. By its action not only on the RhoA/Rho kinase (ROCK) signaling pathway but also on BNP, NO, and cardiac PCSK9 modulation activity. The study provided a promise to use Fasudil clinically in MS beyond RhoA/ROCK inhibition.

Key words: Sucrose; Fasudil; Rho kinase; PCSK9/BNP.
1. **Introduction**

Metabolic syndrome (MS) encompasses multiple disorders that collectively increase the risk of atherosclerotic cardiovascular disease, insulin resistance (IR), diabetes mellitus (DM), with vascular and neurological complications [1]. Unnecessary consumption of high-fat, sucrose, or fructose can encourage the induction of MS. Sucrose increases triglyceride (TG) levels leading to an increase in abdominal fat, inducing IR [2,3]. Moreover, sucrose directly raises the intrahepatic fat via constraining fatty acid oxidation [4]. Additionally, oxidative stress induced by hyperglycemia may impair nitric oxide (NO) production, bioavailability, and responsiveness in the myocardium and blood vessels, resulting in diminished vasodilator capacity and increased platelet aggregation [5].

Natriuretic peptides (NPs) are produced in the heart and regulate fluid homeostasis and blood pressure via diuresis and vasodilation [6]. NPs also regulate glucose homeostasis as well as thermogenesis in adipose tissues. Brain NP (BNP) decreases glucose levels and improves insulin resistance in acute coronary syndrome [7].

Circulating proprotein convertase subtilisin/kexin type 9 (PCSK9) may be indicative of CVS events in patients with coronary artery disease as it effectively correlates with TG levels. Therefore, plasma PCSK9 levels may serve as a valid indicator for CVD risk assessment. It is clear from these signaling pathways that inhibition of PCSK9 releases the endogenous "brake" on low-density lipoprotein cholesterol (LDL) receptor-mediated clearance [8]. PCSK9 is involved in several tissues including aortic endothelial cells (ECs), macrophages, dendritic cells, and epithelial cells. A previous study reported that PCSK9 enhances atherosclerosis by disturbing serum levels of LDL, together with worsening the biological cellular functions in the arterial vasculature, and deteriorating arteriosclerosis [9]. PCSK9 concentration has a direct relationship with the severity of atherosclerosis [10, 11].

Fasudil is a potent reversible Rho kinase enzyme (ROCK) inhibitor. RhoA is one of the serine/threonine protein kinases, and ROCK is the second messenger of guanine triphosphatase enzyme (GTPase) [12]. It inhibits the phosphorylation of myosin-like chain (MLC) kinase, thereby increasing endothelial nitric oxide synthase.
(eNOS) expression and enhancing NO production [13]. Fasudil is the only clinically approved nonselective ROCK inhibitor used to decrease blood pressure [14]. This leads to a plethora of benefits in patients with vasospastic and stable angina, hypertension, pulmonary hypertension, stroke, and chronic heart failure [15].

The current study aimed to investigate the possible benefits of Fasudil on cardiovascular changes and IR in rats with sucrose-induced MS by measuring the serum Rho kinase, cardiac brain natriuretic peptide (BNP), and PCSK9 expression.

2. Materials and Methods

2.1. Materials

- Sucrose (El Nasr Pharmaceutical Chemicals, Cairo, Egypt): Rats received 30% sucrose in their drinking water [16].
- Fasudil powder (250 mg) (Sigma Aldrich Co., USA) [17].

2.2. Study Design

Twenty-four male adult Wistar albino rats weighing between 180 to 230 g were utilized. The rats were procured from the National Research Center animal house, at Cairo University. They were allowed a seven-day adaptation period before the commencement of the study. The rats were divided into four groups, each consisting of 6 rats:

- **Control group**: Rats received orally administered distilled water for 21 days.
- **Fasudil group**: Rats were subcutaneously injected with Fasudil (10 mg/kg daily) [17].
- **Sucrose-fed group**: Rats were provided with 30% sucrose in their drinking water for 21 days [16].
- **Sucrose + Fasudil group**: Rats received both sucrose and Fasudil at the same doses daily for 21 days.

At the end of the 21-day experiment, the rats underwent a 12-hour overnight fasting period, following which the following measurements were taken:

- **Body Weight**: Rat weights were measured using an animal weight scale.
- **Blood Pressure and Heart Rate (BP and HR)**: Blood pressure and heart rate were measured weekly using non-invasive blood pressure ADI instruments (IN125 NIBP, Australia), utilizing specialized tail cuffs.
for pulse and blood pressure measurement [18].

**Blood Sample Collection and Serum Analysis:**

After light anesthesia, 2 ccs of blood were retro-orbitally withdrawn from each rat into tubes with EDTA for the assessment of glycated hemoglobin (HbA1c) using the Suresign Finecare Analyzer and Finecare HbA1C Test Kit (Ballymena, UK). An additional 5 cc blood samples were collected, and centrifuged for 10 min at 3000 rpm, and the separated serum was analyzed for glucose levels using the Rat Glucose Assay kit (CrystalChem, Illinois, USA). The remaining serum was stored at -80°C for subsequent analysis of nitric oxide (NO) levels using the Nitric oxide Assay Kit (Thermo Fisher, Massachusetts, USA), insulin levels using the Rat Insulin ELISA Kit (WuhanFine Biotech, Wuhan, China), and Rho kinase levels using the ROCK Activity Assay Kit – STA-416 (Cell Biolabs, San Diego, USA).

Furthermore, hearts were excised from the sacrificed rats, washed with cold saline, and divided into two parts. One part was preserved in 10% formalin for histopathological examination, while the other part was stored at -80°C for the measurement of brain natriuretic peptide (BNP) and proprotein convertase subtilisin/kexin type 9 (PCSK9) levels using ELISA kits from Sinobiological (Texas, USA).

**2.3. Statistical Methods**

The collected data underwent revision for completeness and logical consistency before being transferred to the Statistical Package for Social Science (SPSS) Version 22 for analysis. Descriptive statistics were employed to express the data (mean ± standard deviation for quantitative variables). The comparison of quantitative variables between groups was conducted using an ANOVA test followed by a post hoc test for inter-group comparison. A *p*-value < 0.05 was considered significant.
3. Results

3.1. Hemodynamic Parameters

**Systolic and Diastolic Blood Pressure (SBP, DBP)**

Fasudil treatment significantly decreased SBP and DBP levels compared to the sucrose group (Table 1). There was a significant decrease in HR among the studied groups, except in the normal control (Table 1).

**Table 1**: Hemodynamic parameters in rats with MS.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>HR beat/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>131.67±2.58</td>
<td>89.67±1.37</td>
<td>436.67±18.62</td>
</tr>
<tr>
<td>Fasudil</td>
<td>103.00±2.68*#</td>
<td>66.33±4.93*#</td>
<td>323.33±22.51*</td>
</tr>
<tr>
<td>Sucrose</td>
<td>166.5±12.33*</td>
<td>110.33±15.60*</td>
<td>361.67±29.94*</td>
</tr>
<tr>
<td>Sucrose + Fasudil</td>
<td>91.33±16.62*#</td>
<td>63.33±13.66*#</td>
<td>380.00±54.41*@</td>
</tr>
</tbody>
</table>

*The data denotes (mean ±SD), * Significant at P < 0.05 compared groups to normal. #  Significant compared to sucrose-treated rats, *@ Significant compared to Fasudil- control group.

3.2. Biochemistry Measurements

**Serum Glucose, Insulin, Glycated Hemoglobin, and LDL-Cholesterol**

There was a significant decrease in serum glucose and insulin values in the Fasudil-treated groups compared to the sucrose group (p < 0.05). Insulin levels significantly increased in the sucrose and Fasudil-treated groups compared to the normal control (p < 0.05). The current data revealed a significant increase in LDL cholesterol levels (p < 0.05) in the sucrose and Fasudil-treated groups compared to the control group. Additionally, the results showed a significant decrease in LDL cholesterol levels (p < 0.05) in the Fasudil-treated group compared to the sucrose group (Table 2).
Table 2: Body weight, glycemic parameters and LDL in rats with experimental MS.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight (g)</th>
<th>Glucose (mmol/L)</th>
<th>Insulin (µIU/L)</th>
<th>HbA1C %</th>
<th>LDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>226.67±4.16</td>
<td>4.55±0.51</td>
<td>8.15±0.29</td>
<td>5.3±1.04</td>
<td>130.23±9.4</td>
</tr>
<tr>
<td>Fasudil</td>
<td>236.67±10.33#</td>
<td>4.91±0.13#</td>
<td>7.79±.81#</td>
<td>4.55±0.06 #</td>
<td>122.33±3.14#</td>
</tr>
<tr>
<td>Sucrose</td>
<td>266.67±12.11*</td>
<td>20.03±2.28*</td>
<td>20.93±1.05*</td>
<td>8.70±0.45*</td>
<td>210.33±9.65*</td>
</tr>
<tr>
<td>Sucrose +</td>
<td>228.33±6.83#</td>
<td>10.53±1.38*#@</td>
<td>12.07±1.53*@</td>
<td>5.97±0.721@</td>
<td>151.33±11.64</td>
</tr>
<tr>
<td>Fasudil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant at P < 0.05 compared to normal group. 
#Significant compared to sucrose set. 
@Significant compared to Fasudil control rats.

**Serum Nitric Oxide (NO)**

The results indicated that levels of NO were significantly higher in all groups compared to the normal control. Additionally, sucrose-treated rats exhibited a significant increase in NO compared to the Fasudil-treated rats (Figure 1a).

**Cardiac Brain Natriuretic Peptide (BNP)**

The results revealed a significant decrease in BNP values in sucrose-treated rats compared to normal rats. Furthermore, the levels of BNP significantly decreased (p < 0.05) in Fasudil + sucrose-treated animals compared to Fasudil-normal rats. Notably, the levels of BNP were significantly elevated in Fasudil-treated rats compared to sucrose-treated rats (p < 0.05) (Figure 1b).

**Serum Rho Kinase Enzyme**

Rho kinase concentration was significantly elevated in the sucrose group compared to other groups. Additionally, Rho kinase levels were increased in the Fasudil-treated groups compared to the control group (p < 0.05) (Table 3, Figure 1c).
Table 3: Effect of Fasudil on serum Rho kinase, nitric oxide, cardiac BNP and PCSK9 in the studied groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Rho-kinase (ng/mL)</th>
<th>NO (nmol/mL)</th>
<th>BNP pg/mg</th>
<th>PCSK9 Pg/mg open</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.903±0.094</td>
<td>13.83±1.56</td>
<td>152.10±8.41</td>
<td>1.005±0.01</td>
</tr>
<tr>
<td>Fasudil</td>
<td>1.90±0.358*</td>
<td>12.17±1.12*#</td>
<td>165.40±2.59*#</td>
<td>1.0133±0.005*#</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.97±1.54*</td>
<td>89.96±10.65*</td>
<td>57.96±5.21*</td>
<td>7.40±1.41*</td>
</tr>
<tr>
<td>Sucrose + Fasudil</td>
<td>3.10±0.71*#@</td>
<td>32.2±3.39*#@</td>
<td>139.23±11.92*#@</td>
<td>2.97±0.226*#@</td>
</tr>
</tbody>
</table>

The data represents mean ± SD. *Significant at P < 0.05 compared versus normal group # Significant compared versus sucrose, @ Significant compared to Fasudil alone group.

Figure 1: Mean levels of a) NO, b) BNP, and c) RHO kinase and PCSK-9 in experimental groups.

Histopathology of Cardiac Tissue

All treated groups showed a marked decrease in both hypertrophied cardiomyocytes and interstitial fibrosis. Sections stained with Hematoxylin and Eosin were examined and graded for hypertrophy, ranging from 0 to 3. The normal control group exhibited grade 0 hypertrophy, indicating no abnormality in the cross diameter of myocytes at the nuclear level (Figure 2a). The sucrose group displayed the maximum myocyte hypertrophy (grade 3) among all studied groups (Figure 2b), while the Fasudil-treated group exhibited the lowest hypertrophy (grade 1) (Figure 2c).

Sections stained with Masson's trichrome were examined and graded for interstitial fibrosis. The control samples showed grade 0, indicating no fibrosis.
(Figure 2d). In contrast, the sucrose samples displayed marked fibrosis grade 1 (Figure 2d). The Fasudil-sucrose-treated rats exhibited minimal fibrosis (Figure 2e).

Figure 2: Histopathology of Cardiac Tissue. a) Normal cardiac section exhibited normally arranged muscle bundles, with central nuclei (H&EX200), b) Cardiac muscle section in sucrose–treated rats display widely separated bundles have dark nuclei with noticeable vacuoles and congestion (H&E × 200), c) Cardiac muscle section in Fasudil+ sucrose–treated rats presenting normal arranged and thickened bundles. The section shows a slight widening of central nuclei and least congestion (H&E × 200), d) Cardiac muscle of sucrose–treated rats displays obvious collagen fibre accumulation (Masson’s trichrome × 200), e) Cardiac muscle of Fasudil + sucrose-treated rats display slight collagen accumulation (Masson’s trichrome × 200).

4. Discussion

Increased sucrose intake in experimental rats results in cardiovascular illness evidenced by augmented mass of the left ventricle, elevated SBP, and metabolic syndrome (MS) induction [19]. Rho/ROCK inhibitors, as shown by previous studies, surge the expression of endothelial nitric oxide synthase (eNOS) in vivo and in vitro, also controlling myosin phosphatase activity. This may be due to stimulation of myosin-binding subunit phosphorylation [20], further increasing the dilatation of the left ventricle and its filling pressure. ROCK inhibitors like Fasudil play an essential role in the cascade of the RhoA-ROCK
pathway after myocardial infarction, resulting in cardiovascular disorders such as myocardial hypertrophy and ventricular remodeling. Inhibiting the pathway of RhoA-ROCK signals could be useful as a therapy for cardiovascular diseases such as hypertension, atherosclerosis, and hypertrophic heart failure [21]. Currently, Fasudil has been shown to lower systolic and diastolic blood pressure as well as heart rate.

Histopathology of the heart displayed minimal fibrosis and cardiac hypertrophy. Along with enhanced insulin sensitivity, there was a reduction in hyperglycemia and hyperinsulinemia in sucrose-treated animals. Insulin normally regulates vascular endothelial nitric oxide (NO), endothelin, and prostaglandin I2. The combination of insulin with its receptors activates the phosphatidylinositol-3 kinase channel, thereby increasing endothelial NOS gene expression and NO release [22]. Fasudil treatment of the sucrose model induced a significant increase in NO compared to normal and Fasudil control groups. It enhanced the endothelium-mediated vasodilator response via inhibition of endothelin-1 (ET-1) and promoting NO synthesis and secretion in endothelial cells [23].

NO concentration was significantly increased in our sucrose-treated rats. This may be due to the early induction of insulin resistance, which increases the expression and activity of eNOS [24]. Significant elevations of serum NO levels in MS patients were reported in previous studies [25]. Acute exposure of the endothelium to high-glucose concentrations up-regulates eNOS expression via activation of protein kinase C [26,27]. NO relaxes vascular smooth muscles and conveys hyperpolarization by regulating large-conductance Ca2+-activated K+ channels (BKCa).

Previous research exhibited an inverse relationship between BNP and risk for Type 2 diabetes. Genetically high BNP levels were associated with increased insulin sensitivity [28]. In our study, Fasudil significantly increased BNP levels. This can guard against the occurrence of insulin resistance and type 2 diabetes mellitus. Silencing of proprotein convertase subtilisin/kexin type 9 (PCSK9) decreases inflammatory cytokines in the aorta, which attenuates plaque inflammation without altering LDL receptor levels. This is due to PCSK9's influence on inflammatory pathways to activate NF-κB and up-regulate Toll-like receptor 4 (TLR4) expression [29]. This can explain the decrease in PCSK9 by Fasudil in our study.

Conclusion

Fasudil conferred a cardioprotective effect in our model of metabolic syndrome,
through improving systolic pressure, glycemic indices, and serum levels of LDL, Rho kinase, NO, and BNP levels. The glucose, insulin, and HbA1C serum levels were diminished in the treated group compared with the sucrose group. Fasudil showed cardio-protection not only via the Rho-kinase pathway but also through its ameliorating actions on PCSK9 and BNP levels. Further research is necessary to elucidate the molecular pathways through which these effects are produced. Clinical confirmation of experimental data and proof of cardio-protection by Fasudil can be undertaken.

**Ethical approval:** The current study was approved by the Ethics Committee of Animal Research of the Faculty of Medicine, Fayoum University, Egypt.

**References**


