CHAPTER 5

RESULTS
5. RESULTS

5.1 DIABETES STUDY

5.1.1 Physiological Parameters

1) Body Weight, Feed Intake & Water Intake

Body weight analysis, revealed insignificant STZ-rutin interaction [F (1, 20) = 1.36, p > 0.05 and η² = 0.02]. But, the main effect of STZ [F (1, 20) = 45.47, p < 0.001, η² = 0.52 and d= 3.6] and rutin [F (1, 20) = 20.32, p < 0.001, η² = 0.23 and d= 2.68] were significant (Fig 5.1A). In case of feed intake, our results revealed that STZ-rutin interaction [F (1, 12) = 18.65, p < 0.001 and η² = 0.35], main effect of STZ [F (1, 12) = 12.55, p < 0.01 and η² = 0.23 and d= 3.17] and main effect of rutin [F (1, 12) = 10.35, p < 0.01 and η² = 0.19 and d= 3.42] were significant (Fig 5.1B). For water intake our results revealed that STZ-rutin interaction [F (1, 12) = 3.44, p < 0.001 and η² = 0.21], main effect of STZ [F (1, 12) = 65.02, p < 0.001, η² = 0.45 and d= 6.04] and main effect rutin [F (1, 12) = 37.26, p < 0.001, η² = 0.25 and d= 4.92] were significant (Fig 5.1C). These results are in line with common symptoms of chronic diabetes, as diabetic animals had significant hyperglycemia, reduced body weight, polyphagia and polydipsia. Rutin was helpful in alleviating all of these parameters and hence controlling diabetes and associated complications.

Figure 5.1: Effect of STZ and rutin treatment on (A) body weight, (B) feed intake and (C) water intake. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]
5.1.2 Biochemical Parameters

1) Fasting Blood Glucose (FBG)

FBG analysis revealed that STZ-rutin interaction [F (1, 20) = 39.73, p < 0.001 and $\eta^2 = 0.23$], main effect of STZ [F (1, 20) = 76.66, p < 0.001 and $\eta^2 = 0.45$ and d = 4.71] and the main effect of rutin [F (1, 20) = 32.9, p < 0.001 and $\eta^2 = 0.19$ and d = 3.56] were significant (Fig 5.2A).

2) Oral Glucose Tolerance Test (OGTT)

At 0 min, STZ-rutin interaction [F (1, 20) = 29.9, p < 0.001 and $\eta^2 = 0.21$], main effect of STZ [F (1, 20) = 69.29, p < 0.001 and $\eta^2 = 0.31$ and d = 4.04] and rutin [F (1, 20) = 23.92, p < 0.0001 and $\eta^2 = 0.16$ and d = 3.02] were observed to be significant. At 15 min, we observed a non-significant STZ-rutin interaction [F (1, 20) = 3.42, p > 0.05 and $\eta^2 = 0.02$], a significant main effect of STZ [F (1, 20) = 73.18, p < 0.001 and $\eta^2 = 0.51$ and d = 3.3] and a significant main effect of rutin [F (1, 20) = 44.53, p < 0.001 and $\eta^2 = 0.31$ and d = 2.53]. At 30 min, we observed a significant STZ-rutin interaction [F (1, 20) = 32.82, p < 0.001 and $\eta^2 = 0.2$], main effect of STZ [F (1, 20) = 54.6, p < 0.001 and $\eta^2 = 0.33$ and d = 4.35] and rutin [F (1, 20) = 54.42, p < 0.001 and $\eta^2 = 0.33$ and d = 3.96]. At 60 min, we observed a significant STZ-rutin interaction [F (1, 20) = 33.39, p < 0.001 and $\eta^2 = 0.27$], main effect of STZ [F (1, 20) = 47.55, p < 0.001 and $\eta^2 = 0.38$ and d = 3.84] and rutin [F (1, 20) = 22.13, p < 0.001 and $\eta^2 = 0.17$ and d = 3.08]. At 120 min of the OGTT, we observed a non-significant STZ-rutin interaction [F (1, 20) = 31.83, p < 0.001 and $\eta^2 = 0.27$], a significant main effect of STZ [F (1, 20) = 38.12, p < 0.001 and $\eta^2 = 0.32$ and d = 3.43] and rutin [F (1, 20) = 27.8, p < 0.001 and $\eta^2 = 0.23$ and d = 3.16] (Fig 5.2B)

3) Serum Insulin

For fasting serum insulin levels, STZ-rutin interaction [F (1, 12) = 87.33, p < 0.001 and $\eta^2 = 0.26$], main effect of STZ [F (1, 12) = 177.9, p < 0.001 and $\eta^2 = 0.53$ and d = 8.6] and the main effect of rutin [F (1, 12) = 52.9, p < 0.001 and $\eta^2 = 0.16$ and d = 7.2] were observed to be significant (Fig 5.2C).

Our results indicate that diabetic animals had significantly higher FBG levels and impaired glucose tolerance. They also had hypoinsulinemia, probably due to the multiple STZ injections. Rutin treatment improved the FBG levels, glucose tolerance and serum insulin levels, thereby exhibiting strong anti-diabetic activity.
Figure 5.2: Effect of STZ and rutin treatment on A) fasting blood glucose (FBG), B) oral glucose tolerance test (OGTT) and C) serum insulin level. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]

5.1.3 Locomotion & muscle coordination

1) Open Field Test (OFT)

Our results demonstrated a significant STZ-rutin interaction [F (1, 20) = 4.44, p < 0.05 and η² = 0.12], the main effect of STZ [F (1, 20) = 4.53, p < 0.05, η² = 0.12 and d = 1.9] and the main effect of rutin treatment [F (1, 20) = 7.99, p < 0.01, η² = 0.22 and d = 2.27] for the number of line crossings in the OFT (Fig 5.3A).
2) Beam Walk Test

For beam walk test (Fig 5.3B), our results revealed a significant STZ-rutin interaction [F (1, 20) = 15.14, p < 0.001 and $\eta^2 = 0.13$], the main effect of STZ [F (1, 20) = 15.14, p < 0.001 and $\eta^2 = 0.13$ and d = 2.18] and the main effect of rutin treatment [F (1, 20) = 68.38, p < 0.001, $\eta^2 = 0.57$ and d = 3.8].

Our results indicate that diabetes impairs locomotion and muscle coordination. Treating animals with rutin rescues these deficits as animal show improved locomotion in the OFT, and excellent muscle coordination in beam walk test.

![Graph A: Open Field Test - No. of line crossings](image1)

**Figure 5.3:** Effect of STZ and rutin treatment on locomotor & muscle coordination parameters. A) Open field-Number of line crossings, and B) beam walk-time taken to cross the beam. [*p < 0.05; **p < 0.01; ***p < 0.001 vs. control. #p < 0.05; ##p < 0.01; ###p < 0.001 vs. STZ]*

5.1.4 Anxiety

1) Elevated Plus Maze (EPM)

For EPM, our results revealed that STZ-rutin interaction [F (1, 20) = 20.75, p > 0.001 and $\eta^2 = 0.11$], the main effect of STZ [F (1, 20) = 129.8, p < 0.001, $\eta^2 = 0.7$ and d= 14.95] and the main effect of rutin treatment [F (1, 20) = 14.6, p < 0.001, $\eta^2 = 0.07$ and d= 8.2] were significant (Fig 5.4A).

2) Open Field Test (OFT)

For, time spent in center, our results revealed that STZ-rutin interaction [F (1, 20) = 19.58, p < 0.001 and $\eta^2 = 0.15$], the main effect of STZ [F (1, 20) = 51.83, p < 0.001, $\eta^2 = 0.4$ and d= 5.3] and the main effect of rutin treatment [F (1, 20) = 36.65, p < 0.001, $\eta^2 = 0.28$ and d= 4.8] were significant (Fig 5.4B). For, number of center entries, our results revealed that STZ-rutin
interaction [F (1, 20) = 21.15, p < 0.001 and η² = 0.34], the main effect of STZ [F (1, 20) = 24.1, p < 0.001, η² = 0.24 and d= 4.2] and the main effect of rutin treatment [F (1, 20) = 34.2, p < 0.001, η² = 0.34 and d = 6.4] were significant (Fig 5.4C). Our results reveal that diabetes led to increased anxiety like behavior and rutin treatment was effective in alleviating it.

Our results indicate that diabetes led to increased anxiety like behavior which was attenuated by rutin treatment as evident by increased time spent in the center of open field and open arm of EPM.

**Figure 5.4:** Effect of STZ and rutin treatment on anxiety presented by A) EPM-% time spent in open arm, B) OFT-time spent in centre, and C) OFT-number of center entries. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]**
5.1.5 Depression

1) Sucrose Preference Test (SPT)

SPT was used to assess anhedonia like behavior. Our results revealed that STZ-rutin interaction \( [F (1, 12) = 7.53, p < 0.05 \text{ and } \eta^2 = 0.15] \), main effect of STZ \([F (1, 12) = 10.7 \ p < 0.01 \text{ and } \eta^2 = 0.21 \text{ and } d = 2.5] \) and main effect of rutin \([F (1, 12) = 20.15, p < 0.001 \text{ and } \eta^2 = 0.4 \text{ and } d = 3.7] \) were significant (Fig 5.5A).

2) Tail Swim Test (TST)

In case of TST immobility time, our results revealed that STZ-rutin interaction \([F (1, 16) = 5.91, p < 0.05 \text{ and } \eta^2 = 0.015] \), main effect of STZ \([F (1, 16) = 36.22, p < 0.001 \text{ and } \eta^2 = 0.09 \text{ and } d = 3.4] \) and main effect of rutin \([F (1, 16) = 334.61, p < 0.001 \text{ and } \eta^2 = 0.85 \text{ and } d = 9.9] \) were significant (Fig 5.5B). Similarly, in case of TST number of upward turnings, our results revealed that STZ -rutin interaction \([F (1, 16) = 37.89, p < 0.001 \text{ and } \eta^2 = 0.34] \), main effect of STZ \([F (1, 16) = 25.19, p < 0.001 \text{ and } \eta^2 = 0.22 \text{ and } d = 5.53] \) and main effect of rutin \([F (1, 16) = 33.37, p < 0.001 \text{ and } \eta^2 = 0.29 \text{ and } d = 5.78] \) were significant (Fig 5.5C).

3) Forced Swim Test (FST)

For FST immobility time, our results revealed that STZ-rutin interaction \([F (1, 20) = 65.58, p < 0.001 \text{ and } \eta^2 = 0.31] \), main effect of STZ \([F (1, 20) = 64.73, p < 0.001 \text{ and } \eta^2 = 0.31 \text{ and } d= 5.28] \) and main effect of rutin \([F (1, 20) = 58.12, p < 0.001 \text{ and } \eta^2 = 0.27 \text{ and } d= 4.97] \) were significant (Fig 5.5D). Diabetic animals showed higher despair and reduced motivation and rutin proved to be an effective antidepressant.

Our results show that diabetes led to severe depressive like behavior with animals having increased despair and anhedonia as evaluated by TST, FST and SPT respectively. Rutin proved to be a potent anti-depressant by not only reducing the depressive behavior in diabetic animals but by increasing the threshold for depression in control animals.
Figure 5.5: Effect of STZ and rutin treatment on depression presented by A) SPT-% sucrose preference, B) TST-immobility time, C) TST-number of upward turns, and D) FST-immobility time. [*p < 0.05, **p < 0.01, ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]

5.1.6 Learning & Memory

1) Passive Avoidance-Step Down (PA-SD) Task

Short-term memory evaluated on day 1 revealed significant STZ-rutin interaction [F (1, 20) = 25.59, p < 0.001 and $\eta^2 = 0.3$], the main effect of STZ [F (1, 20) = 19.01, p < 0.001, $\eta^2 = 0.22$ and $d = 2.77$] and the main effect of rutin treatment [F (1, 20) = 19.45, p < 0.001, $\eta^2 = 0.23$ and $d = 2.79$]. Further, results of memory retention conducted on day 7 (long-term memory retention) of this study demonstrated significant interaction between STZ-rutin [F (1, 20) = 299.5, p < 0.001 and $\eta^2 = 0.25$], main effect of STZ [F (1, 20) = 277.17, p < 0.001, $\eta^2 = 0.23$ and $d = 10.21$] and rutin [F (1, 20) = 559.82, p < 0.001, $\eta^2 = 0.48$ and $d = 29.54$] (Fig 5.6A).
2) Novel Object Recognition (NOR) Test

For NOR test, discrimination index analysis revealed that STZ-rutin interaction \([F (1, 20) = 10.2, p < 0.01\text{ and } \eta^2 = 0.17]\), the main effect of STZ \([F (1, 20) = 12.87, p < 0.001, \eta^2=0.21\text{ and } d= 2.54]\) and the main effect rutin treatment \([F (1, 20) = 17.89, p < 0.001, \eta^2= 0.29\text{ and } d= 2.76]\) were significant (Fig 5.6B).

3) Morris Water Maze (MWM) Test

In MWM, learning was evaluated from day 1 to 4, known as the learning trials. For day 1, our results revealed an insignificant STZ-rutin interaction \([F (1, 20) = 2.43, p > 0.05\text{ and } \eta^2 = 0.04]\) and the main effect of STZ \([F (1, 20) = 0.69, p > 0.05, \eta^2 = 0.01\text{ and } d= 0.44]\). The main effect of rutin \([F (1, 20) = 34.22, p < 0.001, \eta^2 = 0.59\text{ and } d = 3.05]\) was observed to be significant. For day 2, our results revealed that STZ-rutin interaction \([F (1, 20) = 66.31, p < 0.001\text{ and } \eta^2 = 0.23]\), the main effect of STZ \([F (1, 20) = 68.68, p < 0.001, \eta^2 = 0.24\text{ and } d = 5.05]\), and the main effect of rutin \([F (1, 20) = 133.8, p < 0.001, \eta^2 = 0.46\text{ and } d = 6.29]\) were significant. For day 3, our results revealed that STZ-rutin interaction \([F (1, 20) = 132.1, p < 0.001\text{ and } \eta^2 = 0.27]\), the main effect of STZ \([F (1, 20) = 138.45, p < 0.001, \eta^2 = 0.28\text{ and } d = 8.26]\), and the main effect of rutin \([F (1, 20) = 195.35, p < 0.001, \eta^2 = 0.4\text{ and } d = 9.29]\) were significant. For day 4, our results revealed that STZ-rutin interaction \([F (1, 20) = 144.45, p < 0.001\text{ and } \eta^2 = 0.31]\), the main effect of STZ \([F (1, 20) = 120.91, p < 0.001, \eta^2 = 0.26\text{ and } d = 7.47]\), and the main effect of rutin \([F (1, 20) = 184.55, p < 0.001, \eta^2 = 0.39\text{ and } d = 8.5]\) were significant (Fig 5.6C). Memory index was evaluated using number of platform crossings (probe trial) and time spent in the platform quadrant. For probe trial, our results reveal a significant STZ-rutin interaction \([F (1, 20) = 15.48, p < 0.001\text{ and } \eta^2 = 0.21]\). The main effect of STZ \([F (1, 20) = 18.17, p < 0.001, \eta^2=0.24\text{ and } d= 3.1]\) and rutin treatment \([F (1, 20) = 21.07, p < 0.001, \eta^2 = 0.28\text{ and } d= 4.74]\) were observed to be significant (Fig 5.6D). For time spent in platform quadrant, our results reveal that STZ-rutin interaction \([F (1, 20) = 8.57, p < 0.01\text{ and } \eta^2 = 0.15]\), the main effect of STZ \([F (1, 20) = 2.07, p > 0.05, \eta^2=0.03\text{ and } d= 2.64]\) and the main effect of rutin treatment \([F (1, 20) = 24.15, p < 0.001, \eta^2 = 0.44\text{ and } d = 3.75]\) were significant (Fig 5.6E).

Our results demonstrated that diabetes impaired short-term as well as long-term memory retrieval in mice. Additionally, diabetes caused significant cognitive decline by deteriorating learning and memory abilities. Rutin treated diabetic animals prevented any such memory deficit and hence preventing cognitive decline (Fig 5.6).
Figure 5.6: Learning and memory. A) PASD task, B) NOR-discrimination index, C) MWM-learning, D) MWM-probe trial and E) MWM-time spent in platform quadrant. [*p < 0.05; **p < 0.01; ***/aaa p < 0.001 versus control. #p < 0.05; ###p < 0.01; ####/ββ β p < 0.001 versus STZ].
5.1.7 Neurodegeneration

Neurodegeneration and neuronal morphology was evaluated by spine density (number of spines/10μm at 1000 X) and dendritic arborization (number of branches reaching 100 μm at 400 X) through Golgi-cox staining. For spine density, our results reveal that STZ-rutin interaction \([F (1, 12) = 9.8, p < 0.01 \text{ and } \eta^2 = 0.23]\), the main effect of STZ \([F (1, 12) = 12.9, p < 0.01, \eta^2 = 0.31 \text{ and } d= 3.43]\), and the main effect of rutin \([F (1, 12) = 7.1, p < 0.01, \eta^2 = 0.17 \text{ and } d= 3.74]\) were found to be significant (Fig 5.7B). For dendritic arborization, our results reveal that STZ-rutin interaction \([F (1, 12) = 19.65, p < 0.001 \text{ and } \eta^2 = 0.22]\), the main effect of STZ \([F (1, 12) = 24.55, p < 0.001, \eta^2 = 0.28 \text{ and } d= 5.4]\), and the main effect of rutin \([F (1, 12) = 29.99, p < 0.001, \eta^2 = 0.34 \text{ and } d= 5.6]\) were found to be significant (Fig 5.7C).

Our results show that diabetes inflicted severe hippocampal neurodegeneration, especially in the CA3 region. Rutin treatment rescued the neurons from this threat and their morphology appeared similar to control. In diabetes, neurons were short and shrunk, had less networking, and significantly lower spine density. Rutin treated neurons were healthy with extensive networking and significantly higher spine density, compared to untreated diabetic group (Fig 5.7A).

![Figure 5.7: Effect of STZ and rutin treatment on hippocampal neurodegeneration. A) Golgi-cox stained neurons at 400 X and 1000 X, B) spine density, C) dendritic arborization. [*p < ***p < 0.001 versus control; ###p < 0.001 versus STZ] (42)
5.1.8 Protein Expression

1) Western Blot

For insulin, we observed a significant STZ-rutin interaction [F (1, 12) = 22.41, p < 0.001 and η² = 0.21], main effect of STZ [F (1, 12) = 32.6, p < 0.001, η²=0.29 and d= 7.19] and main effect of rutin [F (1, 12) = 42.04, p < 0.001, η²= 0.38 and d= 5.68] (Fig 5.8B). For IR expression (Fig 5.8C), our results revealed an insignificant STZ-rutin interaction [F (1, 12) = 2.01, p > 0.05 and η² = 0.01]. Main effect of STZ [F (1, 12) = 10.55, p < 0.01, η²= 0.07 and d= 1.17] and rutin [F (1, 12) = 116.27, p < 0.001, η²= 0.83 and d= 5.86] were found to be significant. For GLUT4 expression (Fig 5.8D), our results reveal significant STZ-rutin interaction [F (1, 12) = 4.95, p < 0.05 and η² = 0.05]. Main effect of STZ [F (1, 12) = 5.87, p < 0.05, η²=0.065 and d= 0.11] and rutin [F (1, 12) = 67.39, p < 0.001, η²= 0.74 and d= 2.27] were found to be significant. These results indicate that diabetes leads to reduced hippocampal insulin expression, although no change was observed in IR and GLUT4 expression. Rutin treatment not only increased insulin expression but also upregulated IR and GLUT 4 expression (Fig 5.8).

Figure 5.8: Effect of STZ and rutin treatment on A) the hippocampal immunoblot analysis, relative expression of B) insulin, C) insulin receptor, and D) GLUT4. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ]
2) Immunofluorescence

Immunofluorescence was measured using corrected total cell fluorescence (CTCF) (Fig 5.9A). For hippocampal InR (Fig 5.9B), our results showed insignificant STZ-rutin interaction \([F (1, 12) = 3.54, p > 0.07 \text{ and } \eta^2 = 0.07]\) and the main effect of STZ \([F (1, 12) = 3.78, p > 0.05, \eta^2 = 0.07 \text{ and } d = 2.54]\). The main effect of rutin \([F (1, 12) = 29.16, p < 0.001, \eta^2 = 0.6 \text{ and } d = 2.04]\) was observed to be significant. For GLUT4 (Fig 5.9C), STZ-rutin interaction \([F (1, 12) = 0.008, p > 0.05 \text{ and } \eta^2 = 8.68\times10^{-5}]\), the main effect of STZ \([F (1, 12) = 0.96, p > 0.05, \eta^2 = 0.01 \text{ and } d = 0.53]\) were observed to be significant. The main effect of rutin \([F (1, 12) = 79.139, p < 0.001, \eta^2 = 0.85 \text{ and } d = 5.04]\) was found to be significant.

In these results we observed central IR like state with increased InR fluorescence without any change in GLUT4 fluorescence. Rutin treatment upregulated InR and GLUT4 in both control and diabetic state, suggesting a direct role in modulating central insulin signalling.

**Figure 5.9:** Effect of STZ and rutin on the expression of insulin receptor (InR) and glucose transporter 4 (GLUT4) in the hippocampal CA3 region. (A) Immunofluorescence images at 100 X magnification with DAPI (blue, nucleus), FITC (green, InR), and TRITC (red, GLUT4). Corrected total cell fluorescence (CTCF) of (B) InR, and (C) GLUT4. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus STZ].
5.2 CUS STUDY

5.2.1 Physiological Parameters

1) Body Weight, Feed Intake & Water Intake

For body weight changes (Fig 5.10A), our results revealed a significant CUS-rutin interaction \[ F(1, 16) = 46.5, p < 0.001 \text{ and } \eta^2 = 0.39 \], the main effect of CUS \[ F(1, 16) = 12.6, p < 0.001, \eta^2 = 0.108 \text{ and } d = 7.4 \] and the main effect of rutin treatment \[ F(1, 16) = 41.24, p < 0.001, \eta^2 = 0.354 \text{ and } d = 6.01 \]. For feed intake, our results reveal an insignificant CUS-rutin interaction \[ F(1, 16) = 4.15, p > 0.05 \text{ and } \eta^2 = 0.005 \] and the main effect of CUS \[ F(1, 16) = 0.66, p > 0.05, \eta^2 = 0.0008 \text{ and } d = 2.05 \]. However, a significant main effect of rutin treatment \[ F(1, 16) = 783.8, p < 0.001, \eta^2 = 0.974 \text{ and } d = 11.43 \] was observed (Fig 5.10B). Results of water intake (Fig 5.10C) demonstrated an insignificant CUS-rutin interaction \[ F(1, 12) = 5.56, p > 0.05 \text{ and } \eta^2 = 0.168 \], the main effect of CUS \[ F(1, 12) = 1.51, p > 0.05, \eta^2 = 0.458 \text{ and } d = 1.28 \] and the main effect of rutin treatment \[ F(1, 12) = 0.29, p > 0.05, \eta^2 = 0.0089 \text{ and } d = 0.769 \]. Overall, CUS leads to a significant weight reduction, which is alleviated by rutin treatment. Further, rutin treatment increases feed intake in both groups, while the water intake shows insignificant change.

Figure 5.10: Effect of CUS and rutin treatment on body weight (A), feed intake per gram body weight (B) and water intake (C). [\*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]
5.2.2 Locomotion & Muscle Coordination

1) Open Field Test

Our results demonstrated a significant CUS-rutin interaction \([F (1, 20) = 24.33, \ p < 0.001 \text{ and } \eta^2 = 0.202], \) the main effect of CUS \([F (1, 20) = 40.86, \ p < 0.001, \eta^2 = 0.34 \text{ and } d = 6.19] \) and the main effect of rutin treatment \([F (1, 20) = 35.1, \ p < 0.001, \eta^2 = 0.290 \text{ and } d = 6.64] \) for the number of line crossings in the OFT (Fig 5.11A).

2) Beam Walk Test

For beam walk test (Fig 5.11B), our results revealed a significant CUS-rutin interaction \([F (1, 16) = 10.17, \ p < 0.01 \text{ and } \eta^2 = 0.102], \) the main effect of CUS \([F (1, 16) = 3.02, \ p < 0.05, \eta^2 = 0.03 \text{ and } d = 1.59] \) and the main effect of rutin treatment \([F (1, 16) = 70.66, \ p < 0.001, \eta^2 = 0.71 \text{ and } d = 5.3].\)

Our results indicate that chronic stress impairs locomotion and muscle coordination. Treating animals with rutin rescues these deficits as animal show improved locomotion in the OFT, and excellent muscle coordination in beam walk test.

\[ \text{Figure 5.11: Effect of CUS and rutin treatment on locomotor & muscle coordination parameters A) open field test-Number of line crossings, and B) beam walk-time taken to cross the beam. \[ *p < 0.05; **p < 0.01; ***p < 0.001 \text{ vs. control. #p < 0.05; ##p < 0.01; ###p < 0.001 vs. CUS] } \]

(46)
5.2.3 Anxiety

1) Elevated Plus Maze (EPM)

Our results revealed an insignificant CUS-rutin interaction \([F (1, 20) = 0.16, p > 0.05 \text{ and } \eta^2 = 0.003]\) and the main effect of rutin treatment \([F (1, 20) = 1.16, p > 0.05, \eta^2 = 0.023 \text{ and } d = 1.02]\) in the EPM task. However, the main effect of CUS \([F (1, 20) = 28.06, p < 0.001, \eta^2 = 0.57 \text{ and } d = 3.29]\) was observed to be significant (Fig 5.12A).

2) Open Field Test (OFT)

Time spent and number of entries in the center of the OF were also used to evaluate anxiety levels in mice. For time spent in the center (Fig 5.12B), our results reveal significant CUS-rutin interaction \([F (1, 20) = 22.67, p < 0.001 \text{ and } \eta^2 = 0.39]\), the main effect of CUS \([F (1, 20) = 8.9, p < 0.001, \eta^2 = 0.015 \text{ and } d = 4.62]\) and the main effect of rutin treatment \([F (1, 20) = 14.36, p < 0.001, \eta^2 = 0.25 \text{ and } d = 2.72]\). In case of the number of center entries (Fig 5.12C), our results reveal significant CUS-rutin interaction \([F (1, 20) = 28.45, p < 0.001 \text{ and } \eta^2 = 0.32]\), the main effect of CUS \([F (1, 20) = 28.5, p < 0.001, \eta^2 = 0.32 \text{ and } d = 3.65]\) and the main effect of rutin treatment \([F (1, 20) = 10.73, p < 0.001, \eta^2 = 0.122 \text{ and } d = 3.29]\). These results suggest that chronic stress induces anxiety-like behavior in mice. Treating animals with rutin produces anxiolytic effect, and animals were observed to freely explore the OF and EPM.

![Graph A: Elevated Plus Maze](image)

![Graph B: Open Field Test](image)

![Graph C: Open Field Test](image)

**Figure 5.12:** Effect of CUS and rutin treatment on anxiety presented by A) EPM-% time spent in open arm, B) OFT-time spent in centre, and C) OFT-number of center entries. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS*]
5.2.4 Depression

1) Sucrose Preference Test (SPT)

For SPT (Fig 5.13A), subjecting animals to 21 day CUS, resulted in significant CUS-rutin interaction \[ F(1, 8) = 8.05, \ p < 0.05 \text{ and } \eta^2 = 0.09 \], the main effect of CUS \[ F(1, 8) = 19.61, \ p < 0.01, \eta^2 = 0.22 \text{ and } d = 4.42 \] and the main effect of rutin treatment \[ F(1, 8) = 53.77, \ p < 0.001, \eta^2 = 0.06 \text{ and } d = 6.2 \].

2) Tail Swim Test (TST)

In TST depressive behavior was evaluated in the form of immobility time and number of upward turns. In case of immobility time (Fig 5.13B), our results revealed a significant CUS-rutin interaction \[ F(1, 16) = 6.78, \ p < 0.05 \text{ and } \eta^2 = 0.056 \], the main effect of CUS \[ F(1, 16) = 1.46, \ p < 0.05 \text{ and } \eta^2 = 0.012 \text{ and } d = 1.46 \] and the main effect of rutin \[ F(1, 16) = 95.96, \ p < 0.001 \text{ and } \eta^2 = 0.798 \text{ and } d = 3.43 \]. In case of number of upward turnings (Fig 5.13C), our results revealed significant CUS-rutin interaction \[ F(1, 16) = 4.6, \ p < 0.05 \text{ and } \eta^2 = 0.187 \], the main effect of CUS \[ F(1, 16) = 0.51, \ p < 0.05 \text{ and } \eta^2 = 0.021 \text{ and } d = 1.36 \] and the main effect of rutin treatment \[ F(1, 16) = 3.45, \ p < 0.05 \text{ and } \eta^2 = 0.14 \text{ and } d = 2.72 \].

3) Forced Swim Test (FST)

 Immobility time in FST (Fig 5.13D) revealed a significant CUS-rutin interaction \[ F(1, 16) = 19.41, \ p < 0.001 \text{ and } \eta^2 = 0.35 \], the main effect of CUS \[ F(1, 16) = 6.97, \ p < 0.01 \text{ and } \eta^2 = 0.126 \text{ and } d= 4.14 \] and the main effect of rutin \[ F(1, 16) = 5.43, \ p < 0.05 \text{ and } \eta^2 = 0.17 \text{ and } d= 1.6 \].

Our results indicate that CUS-induced significant depression and despair in CUS subjected mice, as evident by reduced preference for sweetened water, increased immobility and reduced upward turns. Rutin not only proved to be an effective antidepressant for stressed animals, but also increased the threshold for depression in control animals.
Figure 5.13: Effect of CUS and rutin treatment on depression presented by A) SPT-% sucrose preference, B) TST-immobility time, C) TST-number of upward turns, and D) FST-immobility time. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

5.2.5 Learning & Memory

1) Passive Avoidance-Step through (PA-ST) Task

Short-term memory evaluated on day 1 revealed significant CUS-rutin interaction [F (1, 16) = 50.62, p < 0.001 and η² = 0.41], the main effect of CUS [F (1, 16) = 23.15, p < 0.001, η² = 0.19 and d = 5.65] and the main effect of rutin treatment [F (1, 16) = 31.23, p < 0.001, η² = 0.26 and d = 6.43]. Further, results of memory retention conducted on day 5 (long-term memory retention) of this study demonstrated significant interaction between CUS-rutin [F (1, 16) = 97.65, p < 0.001 and η² = 0.23], main effect of CUS [F (1, 16) = 93.06, p < 0.001, η² = 0.22 and d = 6.54] and rutin [F (1, 16) = 217.7, p < 0.001, η² = 0.51 and d = 26.12] (Fig 5.14A).
2) Passive Avoidance-Step Down (PA-SD) Task

Short-term memory evaluated on day 1 revealed significant CUS-rutin interaction [$F(1, 16) = 14.33, p < 0.001$ and $\eta^2 = 0.36$], the main effect of CUS [$F(1, 16) = 4.695, p < 0.05, \eta^2 = 0.11$ and $d = 2.13$] and the main effect of rutin treatment [$F(1, 16) = 4.83, p < 0.05, \eta^2 = 0.12$ and $d = 2.32$]. Further, results of memory retention conducted on day 5 (long-term memory retention) of this study demonstrated significant interaction between CUS-rutin [$F(1, 16) = 93.57, p < 0.001$ and $\eta^2 = 0.28$], main effect of CUS [$F(1, 16) = 61.42, p < 0.001, \eta^2 = 0.19$ and $d = 8.59$] and rutin [$F(1, 16) = 153.9, p < 0.001, \eta^2 = 0.47$ and $d = 28.28$] (Fig 5.14B).

Post hoc evaluation demonstrated that CUS impaired short-term as well as long-term memory retrieval in mice. Rutin treated CUS animals prevented any such memory deficit and hence preventing cognitive decline (Fig 5.14).

**Figure 5.14:** Effect of CUS and rutin treatment on learning and memory. A) PAST task, and B) PASD task. [***/aaa $p < 0.001$ versus control group, and ###/βββ $p < 0.001$ versus CUS group].
3) Novel Object Recognition (NOR) Test

Memory was evaluated in terms of discrimination index (preference between novel and familiar objects). Our results revealed an insignificant CUS-rutin interaction \[ F(1, 20) = 2.85, p > 0.05 \text{ and } \eta^2 = 0.035 \]. The main effect of CUS \[ F(1, 20) = 33.18, p < 0.001, \eta^2 = 0.41 \text{ and } d = 2.9 \] and rutin treatment \[ F(1, 20) = 25.16, p < 0.001, \eta^2 = 0.31 \text{ and } d = 2.65 \] were observed to be significant (Fig 5.15A).

4) Morris Water Maze (MWM) Test

Spatial memory was assessed using MWM test. Learning was evaluated from day 1 to 4, known as the learning trials. For day 1, our results revealed an insignificant CUS-rutin interaction \[ F(1, 20) = 0.21, p > 0.05 \text{ and } \eta^2 = 0.002 \] and the main effect of CUS \[ F(1, 20) = 0.02, p > 0.05, \eta^2 = 0.0002 \text{ and } d = 0.24 \]. The main effect of rutin \[ F(1, 20) = 73.77, p < 0.001, \eta^2 = 0.784 \text{ and } d = 3.51 \] was observed to be significant. For day 2, our results revealed that CUS-rutin interaction \[ F(1, 20) = 14.13, p < 0.001 \text{ and } \eta^2 = 0.27 \], the main effect of CUS \[ F(1, 20) = 3.18, p < 0.0001, \eta^2 = 0.06 \text{ and } d = 1.73 \], and the main effect of rutin \[ F(1, 20) = 13.8, p < 0.001, \eta^2 = 0.27 \text{ and } d = 2.64 \] were significant. For day 3, our results revealed that CUS-rutin interaction \[ F(1, 20) = 7.6, p < 0.05 \text{ and } \eta^2 = 0.25 \], the main effect of CUS \[ F(1, 20) = 18.45, p < 0.001, \eta^2 = 0.28 \text{ and } d = 6.26 \], and the main effect of rutin \[ F(1, 20) = 21.83, p < 0.001, \eta^2 = 0.4 \text{ and } d = 7.29 \] were significant. For day 4, our results revealed that CUS-rutin interaction \[ F(1, 20) = 35.21, p < 0.001 \text{ and } \eta^2 = 0.25 \], the main effect of CUS \[ F(1, 20) = 45.91, p < 0.001, \eta^2 = 0.62 \text{ and } d = 5.63 \], and the main effect of rutin \[ F(1, 20) = 65.32, p < 0.001, \eta^2 = 1.2 \text{ and } d = 6.9 \] were significant (Fig 5.15B).

Memory index was evaluated using number of platform crossings (probe trial) and time spent in the platform quadrant. For probe trial (Fig 5.15C), our results revealed a significant CUS-rutin interaction \[ F(1, 16) = 8.29, p < 0.01 \text{ and } \eta^2 = 0.15 \], main effect of CUS \[ F(1, 16) = 22.09, p < 0.001, \eta^2 = 0.39 \text{ and } d = 3.3 \] and main effect of rutin \[ F(1, 16) = 10.14, p < 0.01, \eta^2 = 0.18 \text{ and } d = 3.2 \]. For time spent in platform quadrant, our results revealed an insignificant CUS-rutin interaction \[ F(1, 16) = 2.95, p > 0.05 \text{ and } \eta^2 = 0.089 \]. Main effect of CUS \[ F(1, 16) = 1.59, p > 0.05, \eta^2 = 0.05 \text{ and } d = 1.61 \] was insignificant too. Although the main effect of rutin \[ F(1, 16) = 12.14, p < 0.01, \eta^2 = 0.38 \text{ and } d = 2.17 \] was significant (Fig 5.15D).

These findings suggest that chronic stress impairs memory, and that rutin treatment efficiently alleviates the memory dysfunction.

(51)
Figure 5.15: Learning and memory. A) NOR-discrimination index, B) MWM-learning, C) MWM-probe trial and D) MWM-time spent in platform quadrant. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]*

5.2.6 Histopathological Evaluation (Hematoxylin & Eosin (H&E) Staining)

Integrity of the hippocampal neurons was determined by H&E staining of the 5 µm thick coronal hippocampal section. Neuronal damage was evaluated in CA1, CA2 and CA3 region of the hippocampus in terms of the number of viable neurons observed in one field of the microscope at 400 X magnification (Fig 5.16A). Our results revealed an insignificant CUS-rutin interaction \[ F(1, 16) = 2.48, p > 0.05 \text{ and } \eta^2 = 0.09 \], the main effect of CUS \[ F(1, 16) = 4.25, p > 0.05, \eta^2 = 0.16 \text{ and } d = 2.18 \] and the main effect of rutin treatment \[ F(1, 16) = 3.53, p > 0.05, \eta^2 = 0.13 \text{ and } d = 2.85 \] in CA1 region (Fig 5.16B). Neuronal integrity analysis in the CA2 region revealed a significant CUS-rutin interaction \[ F(1, 16) = 9.07, p < 0.01 \text{ and } \eta^2 = 0.16 \] and the main effect of rutin treatment \[ F(1, 16) = 29.18, p < 0.001, \eta^2 = 0.53 \text{ and } d = 3.37 \]. However, the main effect of CUS \[ F(1, 16) = 1.01, p > 0.05, \eta^2 = 0.018 \text{ and } d = 1.16 \] was insignificant (Fig 5.16C). A significant CUS-rutin interaction \[ F(1, 16) = 67.76, p < 0.001 \text{ and } \eta^2 = 0.42 \], the main effect of CUS \[ F(1, 16) = 16.5, p < 0.001, \eta^2 = 0.01 \text{ and } d = 4.75 \] and
the main effect of rutin treatment \([F (1, 16) = 76.5, p < 0.001, \eta^2 = 0.47 \text{ and } d = 7.01]\) was observed in the CA3 region of the hippocampus (Fig 5.16D). Although, majority of the hippocampus was intact, and did not show any significant damage, we observed that CUS-induced marked damage in the CA3 region. Rutin treatment alleviated this stress-mediated hippocampal neuronal loss.

![Image](image_url)

**Figure 5.16**: Effect of CUS and rutin treatment on hippocampal integrity. Images depict hematoxylin and eosin-stained sections (5 μm) of different regions of hippocampus, and the number of cells at 400X magnification. Results are depicted as mean ± SD \((n = 4)\). [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]*

5.2.7 Biochemical Parameters

1) Fasting blood glucose (FBG)

FBG revealed a significant CUS-rutin interaction \([F (1, 20) = 11.66, p < 0.01 \text{ and } \eta^2 = 0.097]\), main effect of CUS \([F (1, 20) = 60.57, p < 0.001 \text{ and } \eta^2 = 0.5 \text{ and } d = 4.18]\) and main effect of rutin \([F (1, 20) = 28.29, p < 0.001 \text{ and } \eta^2 = 0.23 \text{ and } d = 3.29]\). These results indicate that CUS was associated with a significant increase in FBG leading to a development of a pre-diabetic
state in mice. Rutin treatment prevented the development of pre-diabetes and hence showed significant anti-diabetic effect (Fig 5.17A).

2) Oral Glucose Tolerance Test (OGTT)

A two way analysis of the data revealed a significant CUS-rutin interaction at 0 min $[F(1, 20) = 17.58, p < 0.001$ and $\eta^2 = 0.19]$, main effect of CUS $[F(1, 20) = 23.62, p < 0.001$ and $\eta^2 = 0.26$ and $d = 3.93]$ and rutin $[F(1, 20) = 27.47, p < 0.0001$ and $\eta^2 = 0.31$ and $d = 3.87]$. At 30 min of the OGTT we observed a non-significant CUS-rutin interaction $[F(1, 20) = 1.71, p > 0.05$ and $\eta^2 = 0.01]$, a significant main effect of CUS $[F(1, 20) = 70.66, p < 0.001$ and $\eta^2 = 0.55$ and $d = 4.07]$ and rutin $[F(1, 20) = 35.68, p < 0.001$ and $\eta^2 = 0.27$ and $d = 2.32]$. Two way analysis of OGTT at 60 min time revealed a significant CUS-rutin interaction $[F(1, 20) = 10.61, p < 0.01$ and $\eta^2 = 0.11]$, main effect of CUS $[F(1, 20) = 35.55, p < 0.001$ and $\eta^2 = 0.39$ and $d = 3.76]$ and rutin $[F(1, 20) = 24.53, p < 0.001$ and $\eta^2 = 0.27$ and $d = 2.56]$. At 120 min of the OGTT, we observed a non-significant CUS-rutin interaction $[F(1, 20) = 46.23, p < 0.001$ and $\eta^2 = 0.28]$, a significant main effect of CUS $[F(1, 20) = 58.32, p < 0.001$ and $\eta^2 = 0.36$ and $d = 5.82]$ and rutin $[F(1, 20) = 34.93, p < 0.001$ and $\eta^2 = 0.21$ and $d = 4.52]$ (Fig 5.17B).

3) Serum Insulin level

Two-way ANOVA of the insulin levels revealed a significant CUS-rutin interaction $[F(1, 12) = 87.33, p < 0.001$ and $\eta^2 = 0.26]$, a main effect of CUS $[F(1, 12) = 177.9, p < 0.001, \eta^2 = 0.53$ and $d= 8.6]$, and rutin treatment $[F(1, 12) = 52.9, p < 0.001, \eta^2 = 0.16$ and $d= 7.2]$. Post hoc comparison revealed a significant increase in the serum levels of insulin in 21 day chronically stressed animals and treating animals with rutin resulted in a significant lowering in serum insulin levels, when compared to CUS (Fig 5.17C).

4) HOMA-IR index (Homeostatic Model of Assessment for Insulin Resistance)

Development of insulin resistance in the chronically stressed animals was evaluated in terms of HOMA-IR index. Results of the two-way ANOVA revealed a significant CUS-rutin interaction $[F(1, 12) = 86.52, p < 0.001 and \eta^2 = 0.22]$, a main effect of CUS $[F(1, 12) = 215.8, p < 0.001, \eta^2 = 0.55$ and $d = 10.49]$ and rutin treatment $[F(1, 12) = 71.4, p < 0.001, \eta^2 = 0.18$ and $d = 6.4]$. Post hoc comparison of the results of HOMA index suggest that CUS is associated with the development of significant insulin resistance in 21 days, when compared to CTRL. Treating stressed animals with rutin for 21 days significantly lowered the HOMA-IR index when compared to CUS, suggesting that rutin can efficiently improve insulin sensitivity in stressed animals (Fig 5.17D).
5) Serum Cortisol Level

Hypercortisolism is the outcome of stress and we evaluated the effect of CUS and rutin treatment on serum cortisol levels. Results of the serum cortisol levels demonstrated a significant CUS-rutin interaction [F (1, 12) = 121.7, p < 0.001 and $\eta^2 = 0.16$], a main effect of CUS [F (1, 12) = 519.2, p < 0.001, $\eta^2 = 0.69$ and $d = 14.34$], and rutin treatment [F (1, 12) = 95.88, p < 0.001, $\eta^2 = 0.12$ and $d = 8.4$]. Post hoc analysis revealed that chronic stress significantly elevated serum cortisol levels when compared to control. Rutin efficiently lowered the stress levels and serum cortisol levels were found to be significantly lower than CUS (Fig 5.17E).

**Figure 5.17:** Effect of CUS and rutin treatment on A) fasting blood glucose, B) oral glucose tolerance test (OGTT), C) serum insulin levels, D) insulin resistance (HOMA-IR index), and E) serum cortisol levels (A). Results are depicted as mean ± SD (n = 4-6). Statistical significance was determined using one-way ANOVA followed by Dunnett post hoc test) and two-way ANOVA. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]
5.2.8 Protein expression

1) Western Blot

For hippocampal insulin expression (Fig 5.18B) our results showed insignificant CUS-rutin interaction \([F (1, 12) = 0.48, p > 0.05 \text{ and } \eta^2 = 0.04]\), main effect of CUS \([F (1, 12) = 0.44, p > 0.05, \eta^2 = 0.03 \text{ and } d = 0.02]\) and main effect of rutin \([F (1, 12) = 0.31, p > 0.05, \eta^2 = 0.02 \text{ and } d = 0.07]\). For InR (Fig 5.18C), our results revealed a significant CUS-rutin interaction \([F (1, 12) = 5.47, p < 0.05 \text{ and } \eta^2 = 0.19]\), main effect of CUS \([F (1, 12) = 9.38, p < 0.01, \eta^2 = 0.32 \text{ and } d = 2.07]\) and main effect of rutin \([F (1, 12) = 2.54, p < 0.05, \eta^2 = 0.09 \text{ and } d = 1.54]\). In case of GLUT4 (Fig 5.18D), our results reveal significant CUS-rutin interaction \([F (1, 12) = 9.95, p < 0.01 \text{ and } \eta^2 = 0.11]\), main effect of CUS \([F (1, 12) = 2.08, p < 0.05, \eta^2 = 0.024 \text{ and } d = 3.18]\) and main effect of rutin \([F (1, 12) = 64.24, p < 0.001, \eta^2 = 0.73 \text{ and } d = 5.7]\). Our results suggest that CUS led to the development of an InR state in the hippocampus with increased IR and decreased GLUT4 expression, even though no changes in insulin expression were observed. Rutin improved the insulin signaling by decreasing the IR but increasing the GLUT4 expression (Fig 5.18).

**Figure 5.18**: Effect of CUS and rutin treatment on A) the hippocampal immunoblot analysis, relative expression of B) insulin, C) insulin receptor, and D) GLUT4. Results are depicted as mean ± SD \((n = 4)\). Statistical significance was determined using one-way ANOVA followed
by Dunnett post hoc test) and two-way ANOVA. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]

2) Immunofluorescence

Immunofluorescence was measured using corrected total cell fluorescence (CTCF) (Fig 5.19). For hippocampal InR (Fig 5.19B), our results showed insignificant CUS-rutin interaction [F (1, 12) = 1.11, p > 0.05 and η² = 0.02]. Although, the main effect of CUS [F (1, 12) = 17.45, p < 0.001, η² = 0.42 and d = 2.82] and the main effect of rutin [F (1, 12) = 10.3, p < 0.01, η² = 0.25 and d = 2.97] were observed to be significant. For GLUT4 (Fig 5.19C), CUS-rutin interaction [F (1, 12) = 20.39, p < 0.001 and η² = 0.14], the main effect of CUS [F (1, 12) = 4.75, p < 0.05, η² = 0.034 and d = 1.55] and the main effect of rutin [F (1, 12) = 101.9, p < 0.001, η² = 0.73 and d = 6.93] were observed to be significant. We observed central IR with increased InR and reduced GLUT4 fluorescence. Rutin treatment restored this anomaly by downregulating InR and upregulating GLUT4, suggesting a direct role in modulating central insulin signalling.

![Immunofluorescence images](image)

**Figure 5.19:** Effect of CUS and rutin treatment on the expression of insulin receptor (InR) and glucose transporter 4 (GLUT4) in the entire hippocampal CA3 region. (A) Immunofluorescence images at 400 X magnification with DAPI (blue, nucleus), FITC (green, InR), and TRITC (red, GLUT4). Corrected total cell fluorescence (CTCF) of (B) InR, and (C) GLUT4. [*p < 0.05; **p < 0.01; ***p < 0.001 versus control. #p < 0.05; ##p < 0.01; ###p < 0.001 versus CUS]