Chapter - 4

Results
4.1 Analysis of characteristics of experimental animals

4.1.1 Heart weight and Body weight

STZ-administered rats showed characteristic symptoms of diabetes, including polydipsia and polyuria. Despite the increased food intake diabetic rats showed poor body weight gain as compared to controls. In diabetic cardiomyopathy myocardial damage leads to development of cardiac hypertrophy. This process causes increase in heart weight. In order to investigate the presence of cardiac hypertrophy we measured weight of the heart. Heart was excised from experimental animals after termination of experiment and weighed. There was no significant change in the heart weight of control and diabetic rats. Moreover CLG treatment did not alter body weight and heart weight in either control or diabetic animals (Table 7).

4.1.2 Blood glucose

Establishment of hyperglycemic condition was confirmed by examining blood glucose levels. On day 3 after STZ administration increase in blood glucose levels were observed. This hyperglycemic condition persisted till the end of duration of experiment. Data presented in Table 7 show blood glucose levels of experimental animals at 8 weeks after diabetes induction. Blood glucose level in the diabetic rats was significantly higher than vehicle controls (p< 0.001). CLG treatment had no effect on this parameter thus confirming absence of insulin-like blood glucose-lowering effect (Table 7).

4.1.3 Serum insulin levels

Development of STZ induced diabetic model involves STZ mediated β-cell toxicity subsequently leading to insulin deficiency. Hence serum insulin levels were examined by ELISA. STZ induced diabetes led to decrease in the serum insulin levels (p< 0.01) as compared to vehicle control. However, CLG had no effect on this parameter (Table 7).
Table 7: Physiological parameters of the experimental animals. All values are given as mean ± SE, 
(n=6-8/group); **p < 0.01, ***p < 0.001 vs control group.

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<th>Control</th>
<th>CLG</th>
<th>STZ</th>
<th>STZ + CLG</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>334 ± 1.08</td>
<td>335.75 ± 3.38</td>
<td>249 ± 2.38***</td>
<td>238 ± 9.20</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.02 ± 0.03</td>
<td>1.06 ± 0.03</td>
<td>0.96 ± 0.02</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>95.29 ± 7.09</td>
<td>88.83 ± 12.91</td>
<td>541.60 ± 13.85***</td>
<td>550.71 ± 9.57</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>0.30 ± 0.08</td>
<td>0.49 ± 0.08</td>
<td>0.16 ± 0.03**</td>
<td>0.15 ± 0.01</td>
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4.1.4 Serum markers of cardiac damage

4.1.4.1 Cardiac troponin I

Troponin I belongs to a complex of three regulatory proteins (troponin C, troponin I, and troponin T) and is essential for muscle contraction. An increase in serum level of cTnI is highly indicative of myocardial damage. Diabetic rats showed significant increase in cTnI levels (p < 0.001) and was restored to normal by CLG treatment (p < 0.05) (Table 8).

4.1.4.2 CK MB and CK NAC

Creatine kinase is a dimeric molecule composed of immunologically distinct sub units, M and B. Three main isoenzymes have been identified – CK MM (skeletal muscle), CK MB (cardiac muscle), and CK BB (brain). In case of myocardial injury, serum CK MB levels increases significantly. Hence the increased level of CK MB along with elevated levels of total CK i.e. CK NAC is considered as a good indicator of myocardial infarction. Diabetic rats showed significant increase in levels of CK MB and CK Nac. CLG treatment reduced levels of these enzymes (Table 8).
4.1.5 Serum markers of liver damage

In order to verify whether chronic clorgyline treatment had any toxic effect on liver, serum markers of liver damage were examined. In diabetic rats, serum levels of ALT and AST were higher when compared to vehicle control rats (p< 0.05). However, CLG treatment did not alter these parameters. This confirms non toxicity of clorgyline treatment (Table 8).

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<th>Control</th>
<th>CLG</th>
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<tr>
<td>Serum markers of cardiac damage</td>
<td></td>
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<tr>
<td>cTnl (ng/ml)</td>
<td>0.09 ± 0.03</td>
<td>0.05 ± 0.02</td>
<td>0.31 ± 0.06 ***</td>
<td>0.09 ± 0.03 *</td>
</tr>
<tr>
<td>CK MB (IU/L)</td>
<td>65.75 ± 18.36</td>
<td>74 ± 2.19</td>
<td>140.5 ± 17.40 *</td>
<td>73.75 ± 15.31 *</td>
</tr>
<tr>
<td>CK Nac (IU/L)</td>
<td>181.83 ± 47.94</td>
<td>204 ± 37.21</td>
<td>277 ± 20.90</td>
<td>224.8 ± 73</td>
</tr>
<tr>
<td>Serum markers of liver damage</td>
<td></td>
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<tr>
<td>ALT (IU/L)</td>
<td>52.83 ± 4.17</td>
<td>50.20 ± 3.15</td>
<td>88.75 ± 8.93  *</td>
<td>97.29 ± 7.92</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>115.80 ± 3.40</td>
<td>120.67 ± 7.01</td>
<td>157.17 ± 5.26 *</td>
<td>152.13 ± 17.13</td>
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Table 8: Physiological parameters of the experimental animals. All values are given as mean ± SE, (n=6-8/group); *p< 0.05, ***p< 0.001 vs control group and  #p< 0.05 vs STZ group.
4.2 Diabetes up regulates myocardial MAO-A activity

MAO-A activity is attributed to either an increase in the expression of MAO-A protein expression or an increase in the availability of its substrate. Thus we first examined myocardial MAO-A protein expression by western blotting. We observed that diabetes did not alter the expression of myocardial MAO-A protein. However, significant increase in MAO-A activity was observed in the myocardium of diabetic rats (p< 0.01). Further we assessed cardiac levels of MAO-A substrate NE and its catabolic product DHPG. Higher DHPG/NE ratio confirms increased MAO-A activity in heart of diabetic rats as compared to vehicle control rats. CLG treatment showed significant reduction (p< 0.001) in MAO-A activity without altering its protein expression (Fig.14).

Fig. 14: Diabetes induces myocardial MAO-A activity. (A) MAO-A protein expression. Representative western blot image (left) and densitometric analysis (right; n=8/group). (B) MAO-A activity (n=6-8/group) and (C) Quantification of DHPG/NE ratio by HPLC method (n=4/group). All values are given as mean ± SE; ** p< 0.01, ***p< 0.001 vs control group; # p< 0.01, ## p< 0.001 vs STZ group.
4.3 Effect of MAO-A inhibition on cardiac function

4.3.1 Hemodynamic parameters

Diastolic dysfunction is the earliest manifestation of diabetic cardiomyopathy. It is characterized by increased filling pressures, decreased contraction velocity, prolonged relaxation and reduced cardiac output. Existence of these parameters can be confirmed by analyzing hemodynamic parameters using cardiac catheterization (Table 9). Significant increase in MABP, LVSP and LVEDP confirmed increased filling pressures in diabetic animals. CLG treatment restored each of these parameters to normal. Compared to vehicle controls, diabetic rats showed lower values of LV±dp/dt along with higher values of diastolic durations indicating decreased contraction velocity and prolonged relaxation. However, except diastolic duration there was no significant change in these parameters after CLG treatment. Since LVSP is a hemodynamic determinant of LV−dp/dt_{min} value of this parameter was corrected as described previously (Weisfeldt et al., 1974; Slama et al., 2005; Grousset et al., 1984; Ogata et al., 2004). (−dp/dt_{min})/LVSP showed significant decrease in diabetic rats (p< 0.05) and was restored to normal after CLG treatment (p< 0.001).

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<th>Control</th>
<th>CLG</th>
<th>STZ</th>
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<tbody>
<tr>
<td>MABP (mmHg)</td>
<td>72.44 ± 2.91</td>
<td>78.54 ± 4.84</td>
<td>98.07 ± 4.57*</td>
<td>62.96 ± 7.56 ***</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>91.28 ± 3.76</td>
<td>93.43 ± 5.39</td>
<td>114.74 ± 4.41*</td>
<td>74.48 ± 8.12 ***</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>61.40 ± 1.93</td>
<td>68.37 ± 3.96</td>
<td>86.89 ± 3.80*</td>
<td>50.26 ± 8.26 *</td>
</tr>
<tr>
<td>Systolic duration (s)</td>
<td>0.088 ± 0.004</td>
<td>0.076 ± 0.003</td>
<td>0.111 ± 0.006**</td>
<td>0.111 ± 0.007</td>
</tr>
<tr>
<td>Diastolic duration (s)</td>
<td>0.165 ± 0.006</td>
<td>0.125 ± 0.008</td>
<td>0.193 ± 0.008*</td>
<td>0.151 ± 0.009*</td>
</tr>
<tr>
<td>LV+dp/dt_{min} (mmHg/s)</td>
<td>824.38 ± 67.72</td>
<td>744.90 ± 41.15</td>
<td>682.55 ± 34.88</td>
<td>529.78 ± 39.39</td>
</tr>
<tr>
<td>LV−dp/dt_{min} (mmHg/s)</td>
<td>-368.16 ± 61.68</td>
<td>-409.98 ± 22.98</td>
<td>-293.27 ± 10.33</td>
<td>-256.58 ± 22.32</td>
</tr>
<tr>
<td>(LV−dP)/dt_{min}/LVSP</td>
<td>3.33 ± 0.17</td>
<td>4.43 ± 0.26</td>
<td>2.57 ± 0.10*</td>
<td>3.80 ± 0.42 ***</td>
</tr>
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</table>

Table 9: Hemodynamic parameters of the experimental animals. MABP: mean arterial blood pressure. LVSP: Left ventricular systolic pressure. LVEDP: Left ventricular end diastolic pressure. All values are given as mean ± SE, (n=4-8/group); *p< 0.05, **p< 0.01 vs control group; #p< 0.05, ###p< 0.001 vs STZ group.
4.3.2 Electrocardiographic parameters

ECG analysis was carried out to investigate changes in the electrical activity of the heart. At 8 weeks after the induction of diabetes, slower heart rate was evident in diabetic rats as compared to vehicle control rats (p< 0.05). Compared to vehicle control, diabetic rats showed prolongation of QRS interval (p< 0.001), QT interval (p< 0.01) and QTc interval (p< 0.05) indicating development of intraventricular conduction abnormalities. CLG treatment restored each of these parameters to normal (Table 10).

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<th>Control</th>
<th>CLG</th>
<th>STZ</th>
<th>STZ + CLG</th>
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<tr>
<td>Heart rate (BPM)</td>
<td>239.88 ± 8.71</td>
<td>305.41 ± 12.75</td>
<td>199.15 ± 7.97 *</td>
<td>231.58 ± 11.90</td>
</tr>
<tr>
<td>QRS interval (ms)</td>
<td>27.78 ± 0.25</td>
<td>33.50 ± 0.58</td>
<td>35.91 ± 0.28 ***</td>
<td>33.50 ± 1.12 *</td>
</tr>
<tr>
<td>QT interval (ms)</td>
<td>139.58 ± 5.12</td>
<td>110.90 ± 1.59</td>
<td>177.27 ± 7.59 **</td>
<td>152.46 ± 5.41 *</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>203.70 ± 7.05</td>
<td>171.20 ± 4.09</td>
<td>231.36 ± 3.47 *</td>
<td>204.03 ± 5.21 *</td>
</tr>
</tbody>
</table>

Table 10: Electrocardiographic measurements of the experimental animals. BPM: Beats Per Minute. All values are given as mean ± SE, (n=4-8/group); *p< 0.05, **p< 0.01, ***p< 0.001 vs control group; #p< 0.05 vs STZ group.
4.4 Effect of clorgyline treatment on Ca\(^{2+}\) handling proteins

Abnormal intracellular calcium homeostasis is recognized as one of the major pathological changes under diabetic condition. Defective Ca\(^{2+}\) homeostasis in heart can result from alteration in the expression and/or activity of multiple proteins involved in the release and uptake of calcium across both the sarcolemma and the sarcoplasmic reticulum (SR). Hence we next examined the effect of CLG treatment on the proteins involved in cardiac calcium homeostasis. In particular, we examined the levels of SERCA2a, RyR2 and NCX-1 at translational level. Western blot analysis showed remarkable decrease in SERCA2a and NCX-1 levels in heart of diabetic rats. CLG treatment could restore protein level of SERCA2a while it had no effect on expression of NCX-1 protein (Fig.15).

**Fig. 15:** Effect of CLG treatment on proteins involved in calcium homeostasis. (A) SERCA2a and (B) NCX-1 protein expression. Representative western blot image (left) and densitometric analysis (right). All values are given as mean ± SE, (n=3-6/group).
We also analyzed expression of RYR2 in cardiac tissue of experimental animals. Cardiac levels of RYR2 expression did not show significant difference in case of control and diabetic rat treated with/without CLG. This might be the reason for partial improvement in myocardial performance after CLG treatment (Fig. 16).

**Fig. 16:** Effect of CLG treatment on proteins involved in calcium homeostasis. Analysis of RYR2 protein expression by labeling with BODIPY FL-X Ryanodine(left) and Quantification of Integrated optical density (right). All values are given as mean ± SE, (n=3-6/group).
4.5 MAO-A inhibition decreases myocardial oxidative stress in diabetic rats

To investigate whether MAO-A is a relevant source of ROS under diabetic condition, effect of MAO-A inhibition on various oxidative stress markers was examined.

4.5.1 Lipid peroxidation

Excessive ROS production causes peroxidation of unsaturated fatty acids leading to formation of cytotoxic aldehydes such as MDA and 4HNE. To assess lipid peroxidation cardiac levels of 4HNE and MDA were examined by immunofluorescence and HPLC analysis respectively. Diabetic rats exhibited higher levels of myocardial 4 HNE than vehicle control group (p< 0.01) and were restored to normal by CLG treatment (p< 0.01). HPLC analysis showed that MDA levels were increased in myocardium of diabetic rats as compared to vehicle controls and this increase was significantly reduced by CLG administration (p< 0.05) (Fig.17A and 17B).

ALDH2 is the key enzyme responsible for detoxification of toxic aldehydes such as 4-hydroxy-2-nonenal (4-HNE). Previous studies showed that ALDH2 has redox-sensitive thiol group at its active site and thus is prone to an oxidative inactivation (Wang et al., 2011). Hence we hypothesized that inactivation of ALDH2 by oxidative stress might be responsible for increase in 4HNE levels and the partial lowering of the oxidative stress by MAO-A inhibition might reverse this condition. The decrease in the ALDH2 activity was observed in the STZ group as compared to the vehicle control group (p< 0.001). This decrease in ALDH2 activity was significantly restored in diabetic rats treated with the CLG (p< 0.001) (Fig.17C).
Fig. 17: MAO-A contributes to oxidative stress mediated lipid peroxidation. Immunofluorescence staining for 4HNE (left) and Quantification of Integrated optical density (right; n=4/group), (B) Quantification of myocardial MDA levels by HPLC method (n=3/group and (C) ALDH2 activity (n=5-9/group). All values are given as mean ± SE; ** p< 0.01, ***p< 0.001 vs control group; # p< 0.05, ## #p< 0.01, p### 0.01 vs STZ group.
4.5.2 Mitochondrial damage

The major phospholipid components of the mitochondrial membranes are rich in unsaturated fatty acids. Cardiolipin is one such phospholipid which is localized within the inner mitochondrial membrane and plays an important role in the mitochondrial bioenergetics. Previous reports have shown that ROS-induced peroxidation of cardiolipin affects the activity of mitochondrial respiratory chain complexes specifically complex I, III and IV (Paradies et al., 2002). Disturbance in the function of complexes I and III can affect ATP production and exacerbate superoxide production. Hence in the present study complex I activity was measured to analyze whether MAO-A contributed to oxidative stress mediated alteration in complex I activity. The decrease in the complex I activity was observed in the STZ group as compared to the vehicle control group. This decrease in complex I activity was restored after CLG treatment. (Fig. 18)

![Complex I Activity](image)

*Fig.18: Increased cardiac MAO-A activity contributed to mitochondrial damage. Complex I activity in the heart of control and diabetic rats treated with/without CLG. All values are given as mean ± SE, (n=5-9/group).*

It is suggested that mtDNA, owing to its close proximity to the mitochondrial respiratory chain, lack of histone protection, and less DNA repair capacity, is vulnerable to ROS attack. Several studies have proposed an existence of a positive correlation between oxidative stress and mtDNA copy number (Al-Kafaji and Golbahar, 2013; Lee et al., 2000). Thus considering the relevance of MAO-A in myocardial oxidative stress, and damage to mitochondrial ETC complex in diabetic rats, we speculate that MAO-A derived ROS may also provoke mtDNA
damage. We compared the copy number of mtDNA in the heart of control and diabetic rats treated with/ without CLG. As predicted, the mtDNA copy number was significantly elevated in the heart of diabetic rats (p< 0.05) and was found to be reduced after MAO-A inhibition (Fig. 19).

![mtDNA Copy Number](image)

**Fig. 19: Increased cardiac MAO-A activity contributed to mitochondrial damage. Analysis of mtDNA copy number by real-time PCR. All values are given as mean ± SE; *** p< 0.05 vs control group, p## # p < 0.001 vs STZ group. (n=5/group).**

Mitochondrial dynamics is the cyclic process of fusion and fission. It plays key role in maintenance of mitochondrial quality. When mitochondrial damage exceeds repairing capacity of the cell, mitochondrial fission carries out asymmetric division of damaged mitochondria. This process causes segregation of severely damaged mitochondria which can be eventually eliminated through mitophagy. Since the markers of mitochondrial damage were normalized in case of CLG treated diabetic rats, we were eager to know the effect of MAO-A inhibition on the proteins involved in the process of mitochondrial dynamics. Real time PCR analysis showed significant increase in expression of Drp1 and Fis1 in case of heart of diabetic rats, while CLG treatment could restore mRNA levels of these two proteins (Fig.20).
Fig. 20: Increased cardiac MAO-A activity contributed to mitochondrial damage. Analysis of Drp1 and Fis1 gene expression by real-time PCR method. All values are given as mean ± SE; * p< 0.05, ** p< 0.01 vs control group; # p< 0.05 vs STZ group, (n=5-9/group).

4.5.3 SOD

SOD is an antioxidant enzyme. Activity of this enzyme increases as compensatory response to the oxidative stress. Hence we next measured the activity of antioxidant enzyme SOD in cardiac tissues of control and diabetic rats. An increase in the SOD activity was observed in the STZ group as compared to the vehicle control group (p< 0.001). This increase in SOD activity was reduced in diabetic rats treated with the CLG (p< 0.05) (Fig.21).

Fig. 21: MAO-A contributes to diabetes induced oxidative stress. SOD Activity. All values are given as mean ± SE; *** p< 0.01 vs control group, # p< 0.05 vs STZ group, (n=5-9/group).
4.5.4 UCP3

UCP3 is an uncoupling protein present on mitochondrial membrane. During mitochondrial respiration, transfer of electrons across ETC complex to O₂ generates proton gradient across mitochondrial membrane. In coupled respiration, this gradient is utilized by F0F1-ATPase for ATP synthesis. Leakage of protons across the mitochondrial inner membrane via UCP3 uncouples mitochondrial respiration from ATP production. It has been shown that ROS and by-products of lipid peroxidation, specifically 4HNE activates UCP to mitigate mitochondrial oxidative stress (Mailloux and Harper, 2011). Hence UCP3 protein levels were examined to verify the extent of mitochondrial uncoupling and redox stress under diabetic condition. We observed a remarkable increase in myocardial UCP3 protein expression in the diabetic group, while CLG treatment was able to restore its expression to the normal levels (Fig. 22).

![UCP3 Protein expression](image)

**Fig. 22: MAO-A contributes to diabetes induced oxidative stress. UCP3 protein expression.** Representative western blot image (left) and densitometric analysis (right). All values are given as mean ± SE; (n=5-9/group).

4.5.5 Effect of clorgyline treatment on H₂O₂ scavenging proteins

Since the antioxidants catalase, GPx, peroxiredoxin-3 (Prx-3) and peroxiredoxin -5 (Prx-5) are known to specifically scavange H₂O₂ in the cytoplasm and mitochondria respectively, we further measured the activity of catalase and GPx and examined the protein expression of Prx-3 and Prx-5. We observed a decrease in the GPx activity (Fig. 23A) and Prx-3 protein expression (Fig. 23C) and an increase in the catalase activity (Fig. 23B) and Prx-5 protein
expression (Fig. 23D) in the hearts of STZ induced diabetic rats. However, CLG treatment had no effect on these alterations.

**Fig. 23: Clorgyline treatment has no effect on myocardial H$_2$O$_2$ scavenging proteins.** (A) Glutathione peroxidase (GPx) activity (n=5/group), (B) Catalase activity (n=6-9/group), (C) Prx-3 protein expression. Representative western blot image (left) and densitometric analysis (right; n=5/group) and (D) Prx-5 protein expression. Representative western blot image (left) and densitometric analysis (right; n=6/group). All values are given as mean ± SE; *p< 0.05, ***p< 0.001 vs control group.
4.6 MAO-A inhibition prevents apoptosis of cardiac cells in diabetic rats

4.6.1 Bcl2/Bax ratio

We investigated the role of MAO-A in diabetes induced cardiac apoptosis. In particular, we focused on the expression of Bcl2 and Bax proteins. Bcl2 and Bax are homologous proteins that have opposing effects on cell survival and death, with Bcl2 serving to prolong cell survival and Bax acting as an accelerator of apoptosis (Korsmeyer, 1999). Thus Bcl2/Bax ratio serves as one of the important markers of apoptosis. We assessed protein levels of Bcl2 and Bax by western blotting. Diabetic rats showed significant decrease in Bcl2/Bax ratio when compared to controls (p<0.05). Interestingly, after treatment with CLG, Bcl2 and Bax levels were seen to be normalized, resulting in a significantly increased Bcl2/Bax ratio (p<0.05) (Fig. 24).

![Bcl2 and Bax Protein expression](image)

**Fig. 24 : MAO-A contributes to alterations in Bcl2 and Bax protein levels under diabetic condition.**

Bcl2 and Bax protein expression. Representative western blot image (left) and densitometric analysis (right). All values are given as mean ± SE; *p < 0.05 vs control group, #p < 0.05 vs STZ group, (n=7/group).
4.6.2 Release of Cytochrome c from mitochondria to cytoplasm

Cytochrome c is tightly bound to the inner membrane by its association with cardiolipin. Oxidative stress mediated peroxidation of cardiolipin causes the release of this tightly bound cytochrome c from mitochondrial inner membrane to the inter membrane space. Once cytochrome c is released, permeabilization of the outer mitochondrial membrane due to Bax oligomerization allows the movement of cytochrome c from mitochondria to the cytoplasm. This cytochrome c release further activates intrinsic pathway of cell death (Kluck et al., 1997). Since we had confirmed that increased MAO-A activity in diabetic rats contributed to mitochondrial oxidative damage and decrease in Bcl2/Bax ratio, we speculate that MAO-A may participate in activation of intrinsic pathway of cell death via cytochrome c release. To detect release of cytochrome c from mitochondria to cytoplasm, subcellular fractionation of heart tissues followed by western blot analysis of the cytosolic fraction was carried out. Results showed that cytosolic cytochrome c levels were increased in diabetic group as compared to vehicle control. This cytochrome c release could be prevented by MAO-A inhibition (Fig. 25).

![Cytoplasmic levels of cytochrome c](image)

**Fig. 25:** MAO-A promotes release of cytochrome c from mitochondria to cytoplasm under diabetic condition. Analysis of cytosolic cytochrome c level by western blotting. Representative western blot image (left) and densitometric analysis (right) All values are given as mean ± SE, (n=3/group).
4.6.3 Caspase activation

Caspases are important regulators of apoptosis. Release of cytochrome c from mitochondria to cytoplasm causes activation of caspases. Therefore, we analyzed the effect of MAO-A inhibition on caspase activation implicated in the cardiac cell death. Heart of diabetic rats showed significant increase in activities of caspase-9 (p< 0.01) and caspase-3 (p< 0.05) as compared to that of vehicle controls. CLG treatment significantly reduced caspase activation (Fig. 26).

![Caspase Activities](image)

**Fig. 26:** MAO-A participates in caspase activation under diabetic condition. (A) Caspase-9 activity (n=4-7/group) and (B) Caspase-3 activity (n=5-6/group). All values are given as mean ± SE; *p< 0.05, **p< 0.01 vs control group; #p< 0.05, ##p< 0.01 vs STZ group.

4.6.4 DNA fragmentation

Since caspase activation leads to endonuclease mediated DNA fragmentation, further, TUNEL assay was performed to analyze DNA fragmentation in cardiac tissue of experimental animals. Heart of diabetic rats showed significantly higher number of TUNEL-positive nuclei than vehicle control group (p< 0.05). However, diabetic rats treated with CLG showed a significant reduction in the number of TUNEL-positive nuclei as compared to that of untreated diabetic rat (p< 0.05) (Fig. 27).
Fig. 27: MAO-A contributes to DNA fragmentation under diabetic condition. Examination of DNA fragmentation in cardiac tissues using TUNEL staining; Representative TUNEL section shown on left, arrow indicate TUNEL positive nucleus and quantification (right). All values are given as mean ± SE; *p < 0.05 vs control group; # p < 0.05 vs STZ group, (n=3-4/group).

4.7 Effect of MAO-A inhibition on cardiac remodeling process in diabetic rats
Heart compensates for loss of cardiomyocytes occurring due to cell death by increasing the size of existing cardiomyocytes leading to hypertrophy or by filling the gaps with excessive production of the extracellular matrix proteins leading to fibrosis. This initial remodeling process may be beneficial as it helps to maintain cardiac function. However, when these conditions persist for longer time they lead to increase in ventricular mass and volume (Fig. 28). These changes affect cardiac function ultimately causing heart failure. Since we had observed that MAO-A contributed significantly to oxidative stress mediated cardiac cell death, we were interested to know its role in cardiac remodeling process.

Fig. 28: Cardiac remodeling process (Adapted from Biernacka et al., Aging and Disease, 2011)

4.7.1 Structural derangements
Hematoxylin and eosin (H & E) staining of the heart tissue sections revealed structural abnormalities such as degeneration and marked separation of myocardial fibers, congestion and hemorrhages in the epicardium and, accumulation of polymorph nuclear neutrophils (PMN) in the myocardium of diabetic rats. However, these abnormalities were prevented by CLG treatment (Fig. 29).

**Fig. 29: MAO-A inhibition attenuates diabetes-induced changes in myocardial histology.** Representative H&E stained cardiac sections; arrows demonstrate a: separation and b: degeneration of myocardial fiber, (n=6-8/group)

### 4.7.2 Hypertrophy
For characterization of hypertrophic phenotype cardiomyocyte cross-sectional area was measured by WGA-FITC staining method. No significant change in cardiomyocyte cross-sectional areas was observed in case of control and diabetic rats treated with or without clorgyline (Fig. 30).

**Fig. 30: MAO-A inhibition attenuates diabetes-induced cardiac hypertrophy.** Representative of WGA-FITC stained sections (left) and quantification of cross sectional area of cardiomyocytes (right). All values are given as mean ± SE, (n=3-4/group).

The absence of hypertrophic phenotype was further confirmed by examining molecular markers of hypertrophy such as ANP and β-MHC. Protein expression of ANP was examined by immunofluorescence studies and PCR analysis was carried out to assess β-MHC gene expression. Levels of these molecular markers did not show significant alteration in case of control and diabetic rats treated with or without clorgyline (Fig. 31).
**Fig. 31:** MAO-A inhibition attenuates diabetes-induced cardiac hypertrophy. (A) Immunofluorescence staining for ANP (left) and Quantification of Integrated optical density (right),(B) RT-PCR analysis of β-MHC mRNA, β-actin was used as an internal control. All values are given as mean ± SE, (n=3-4/group).
4.7.3 Fibrosis

In diabetes myocardial fibrosis is associated with accumulation of excessive collagen deposition, specifically Type I and III. To assess extent of collagen deposition, Sirius red staining was performed. Diabetes induced a significant increase in myocardial interstitial and perivascular collagen deposition as compared to vehicle control (p< 0.05). This was prevented by CLG treatment (Fig.32).

**Fig. 32: MAO-A inhibition attenuates diabetes-induced cardiac fibrosis.** Representative Sirius red-stained sections (left), collagen appears red as indicated by arrows and quantification (right). All values are given as mean ± SE, *p< 0.05 vs control group, #p< 0.05 vs STZ group,(n=5/group).

Myocardial fibrosis was further confirmed by examining a molecular marker of fibrosis; CTGF in the heart of control and diabetic rats treated with or without clorgyline. Immunofluorescence analysis showed that CTGF expression was higher in the heart of diabetic rats as compared to control and was significantly alleviated when diabetic rats were treated with CLG (p< 0.05) (Fig.33).
Fig. 33: MAO-A inhibition attenuates diabetes-induced cardiac fibrosis. Immunofluorescence staining for CTGF (left) and Quantification of Integrated optical density (right). All values are given as mean ± SE. *p < 0.05 vs STZ group, (n=3/group).