CHAPTER I

Effects of diabetogens (Streptozotocin and Alloxan) on the brain and pancreas: A comparative study
INTRODUCTION

Streptozotocin (STZ) and Alloxan monohydrate (ALX) are commonly used diabetogens in the area of diabetic research. While their selective pancreatic β-cells toxicity is identical, mode of cytotoxicity is different (Lenzen, 2008). STZ, a glucosamine-nitrosourea compound is toxic to β-cells by causing DNA damage though alkylation. DNA damage induces activation of poly ADP-ribosylation, which is involved in the induction of diabetes (Szkudelski, 2001). Structurally, STZ is similar to glucose and acts as glucose analogue which is to be transported into the cells by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to β-cells, since these cells have relatively high levels of GLUT2 (Schnedl et al. 1994; Wang and Gleichmann, 1998). Earlier literature indicates that the type of diabetes and its symptoms differ with the employed dose of STZ and animal and species used (Junod et al. 1967; Brodsky and Logothetopoules, 1969; Rerup, 1970).

ALX has ability to induce ROS formation, resulting in the selective necrosis of β-cells. The ALX molecule is structurally so similar to glucose that the GLUT2 glucose transporter in the β-cell plasma membrane accepts this glucomimetic and transports it into the cytosol (Gorus et al. 1982). ALX does not inhibit the function of the transporter (Elsner et al. 2002) and can therefore selectively enter β-cells in an unrestricted manner (Hammarström et al. 1967; Malaisse et al. 2001).

There are many reports on the effects of these two diabetogens on different organ system. STZ induced diabetes is associated with changes in nerve growth factor (NGF) levels in the brain and pancreas of diabetic rats (Sposato et al. 2007). It impairs
glucose oxidation (Bedoya et al. 1996) and decreases insulin biosynthesis and secretion (Bolaffi et al. 1987, Nukatsuka et al. 1990). ALX induced diabetes causes decline in the brain acetylcholine activity of the diabetic rats (Ahmed and Tarannum, 2009). The action of ALX in the pancreas is preceded by its rapid uptake by the β-cells which have been proposed to be one of the important features determining ALX diabetogenicity (Gorus et al. 1982). Another effect of ALX is the formation of reactive oxygen species (Heikkila et al. 1976) to which pancreatic β-cells are more sensitive in comparison to other cells which protects them against ALX toxicity (Malaisse et al. 1982, Tiedge et al. 1997).

In spite of well-known diabetogenic effects of STZ and ALX, a comparative account of the effect of these diabetogen compounds on the expression of hypothalamic stress hormones (CRH and AVP) and in situ pancreatic response in terms of β-cell function (Insulin, Kir6.2) is not yet investigated. Hence following experiment was conducted to study and compare the effects of two well-known diabetogens induced diabetes on the brain, stress (HPA axis) response and pancreatic responses.

**MATERIALS AND METHODS**

**Experimental design**

A total of 30 adult female mice were randomly divided into three groups (n=10 in each group).

1. **Control group**: Received an i. p. injection of citrate buffer (0.1M, pH 4.5) as vehicle.

2. **STZ treated group**: Single dose of streptozotocin (160 mg/kg body weight, i.p.) freshly dissolved in 0.1 M sodium citrate buffer (pH 4.5) was given to overnight fasted animals.
3. **ALX treated group:** Single dose of alloxan monohydrate (140 mg/kg body weight, i.p.) freshly dissolved in 0.1 M sodium citrate buffer (pH 4.5) was given to overnight fasted animals.

Diabetes was induced as mentioned in the General material and method section and during this period weekly body weight and fasting blood glucose level were monitored from blood samples obtained from the tail vein of the animals. At the end of 5th week, mice (n=6) of all the groups were weighed and sacrificed by decapitation. Blood was collected in microcentrifuge tubes, centrifuged and serum was stored in -20°C until assayed for corticosterone. Blood smear were also prepared for neutrophil/lymphocyte (N/L) ratio, a stress parameter. The brain and pancreas of these mice were also processed for the estimation of total nitrite and nitrate (NO\(_X\)) concentration, antioxidant enzymes activities, MDA level, AR activity, AGEs and AOPP level by the methods as mentioned in earlier section.

The brain and pancreata (n=4) of remaining mice were processed for routine histology (haematoxylin-eosin staining), immunofluorescence of insulin and Kir6.2 in pancreas and CRH as well as AVP in brain.

**Data and statistical analysis**

Results are expressed as mean ± S.E.M. SPSS software (version 16.0) was used for statistical analysis. One way analysis of variance (ANOVA) was performed followed by post hoc analysis with Tukey's test. For each test, a value of P ≤ 0.05 was considered statistically significant.

**RESULTS**

**Body weight, blood glucose level and N/L ratio**

After STZ and ALX administration, animals showed a constant weight loss compared to the control mice. Further, while plasma glucose levels of both the
diabetic groups increased 4-5 fold, N/L ratio was also significantly increased in these mice compared to control (Fig. I-1).

**Nitrate - Nitrite level**

A significant increase in the nitrate- nitrite concentration was observed in the brain and pancreatic tissue of STZ as well as ALX treated groups compared to control group (Fig. I-2).

**Effects on antioxidant enzymes and MDA level**

The activities of antioxidant enzymes (CAT, GPx and SOD) were significantly increased in the brain of STZ treated group, whereas ALX treated group showed decreased CAT and SOD activity while GPx remained unaltered in the brain compared to control. However, brain MDA level increased in both the treated groups compared to control (Fig. I-3). On the other hand, in the pancreatic tissue of both the diabetic groups CAT and GPx activities were decreased and SOD activity was increased compared to control (Fig. I-4). MDA level was also increased significantly in the pancreatic tissue of both the diabetic groups compared to control (Fig. I-4).

**Effects on AR activity, AGEs and AOPP level**

AR activity as well as the levels of AGEs and AOPP was significantly elevated in the brain tissue of both the diabetic groups compared to control (Fig. I-5). However, in the pancreatic tissue AR activity was decreased but AGEs and AOPP level were enhanced in both the diabetic groups compared to their respective controls (Fig. I-6).

**Effect on the endocrine pancreas**

Reduced islet cells density was observed in the pancreatic islets of STZ and ALX treated mice compared to control (Fig. I-7).
Effect on the expression of *ir*-insulin and *ir*-Kir6.2 in the islets

Reduced expression of insulin as well as Kir6.2 was observed in both STZ and ALX induced diabetic groups as compared to control (Fig. I-8, I-9).

Effect on the expression of *ir*-CRH in the PVN region

The immunohistochemical localization of *ir*-CRH showed increased expression in the PVN of STZ as well as ALX treated groups compared to control (Fig. I). The intensity as well as the no. of CRH positive cells was found to be increased significantly in the coronal brain sections of STZ and ALX treated group as compared to vehicle treated control mice (Fig. I-10).

Effect on the expression of *ir*-AVP in the PVN region

The immunohistochemical localization of *ir*-AVP also showed increased expression in the PVN of STZ as well as ALX treated groups compared to control. The intensity of AVP positive cells and their numbers were found to be increased significantly in the brain sections of STZ group which was increased further in ALX treated group as compared to vehicle treated control mice (Fig. I-11).

Effect on serum corticosterone level

Serum corticosterone level was significantly increased in both STZ (P< 0.001) and ALX (P< 0.01) induced diabetic groups compared to vehicle treated control group (Fig. I-12).

DISCUSSION

Our results show that both the diabetogen causes pathological conditions in the brain as well as in endocrine pancreas. Both diabetogens are potent inducer of oxidative and nitrosative stress in brain and in pancreatic tissue but the degree of effect varies in terms of different tissues and parameters. It has been also found that
STZ and ALX induced diabetes activates the HPA axis in terms of both CRH and AVP neuropeptides expression.

Present study demonstrated that a single i.p. injection of STZ (160 mg/kg) and ALX (140 mg/kg) produced diabetes mellitus in the 2\textsuperscript{nd} week and the mice remained in that state upto 5\textsuperscript{th} week. In STZ group maximum increase in the blood glucose level was noted in the 2\textsuperscript{nd} week which was maintained upto 5\textsuperscript{th} week. Whereas in ALX group, gradual increase in the blood glucose level observed upto 2\textsuperscript{nd} week was followed by a relative decline but hyperglycemic state was still maintained.

Altered activities of antioxidant enzymes and AR, increased MDA level, AGEs and AOPP causes oxidative stress. Present findings showed that chronic hyperglycemia leads to the generation of excess reactive oxygen species (ROS), through the mitochondrial electron transport chain and causes tissue damage (Brownlee, 2001). There are several ways through which ROS increases in diabetes, i.e. glycation and decreased activity of enzymic and nonenzymic antioxidants (Asayama et al. 1989; Godin et al. 1988). During diabetes an increased production of ROS through glucose autoxidation and protein glycation has been also reported (Wolff and Dean, 1987; Hunt et al. 1990). The formation of MDA, AGEs and AOPP products during progression of diabetes may play a role in brain and pancreatic damage associated with diabetes. In diabetes, AGEs are found in increased amounts in extracellular matrix (Stitt et al. 1998). On the other hand, intracellular production of AGE precursors can also damage cells by modifying intracellular proteins, plasma proteins and ECM proteins. The binding of receptors of AGEs (RAGE) which were present in macrophages, vascular endothelial cells and vascular smooth muscle cells induces the production of ROS (Miyata et al. 1997; Goldin et al. 2006). Several
studies reported that AR, a key enzyme in the polyol pathway contributes to oxidative stress and accelerates the diabetic complications (Lee and Chung, 1999; Chung et al. 2003).

Nitrate-nitrite level was also significantly increased in brain and pancreatic tissue of both STZ and ALX induced diabetic groups compared to control. It was reported that high glucose may enhance nitric oxide (NO) production through increased expression of eNOS and iNOS (Yang et al. 2010; Zhang et al. 2014). Our results also support the concept that hyperglycemia is associated with high NO level (Adela et al. 2015). N/L ratio, a stress parameter is also found to be increased in both the diabetic groups compared to control.

To measure the extent of pancreatic damage we have carried out immunohistochemical staining of insulin and Kir6.2. Later (the Kir6.2) is the subunit of ATP sensitive K⁺ channel present in pancreatic β-cells and are essential in glucose induced insulin secretion (Inagaki et al. 1996). In both the diabetic groups, reduction in insulin immunoreactivity was observed in pancreatic islets compared to control. Further, expression of ir-insulin and Kir6.2 followed the similar pattern and decreased in both the diabetic groups compared to control. Our findings suggest that histological alterations can be seen in diabetes mellitus condition and these changes could correlate to the specific parameters related to oxidative stress.

In recent years, studies have shown that diabetes and stress are associated with each other but experimental/research findings are less documented. Some studies have suggested that stressful events might affect the onset and/or the metabolic control of diabetes, but findings have often been inconclusive (Llyord et al. 2005). Considering these reports we have also checked whether diabetes activates the HPA axis via altering the hypothalamic stress neuropeptides CRH and AVP.
Hypothalamic-pituitary-adrenal (HPA) axis serves as an intricate pathway of neurobiological stress system (Smith and Vale, 2006; Lanfumey et al. 2008). This stress system contributes to the maintenance of homeostasis which is impacted by various physical and psychological stressors (Atkinson et al. 2010; Kudielka et al. 2004). The HPA axis comprises of hypophysiotropic neurons localized in the medial parvocellular subdivision of the PVN which synthesize and secrete corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). CRH is a neuropeptide involved in the control of stress-related behaviours (Kovacs and Sawchenko, 1996). However, CRH in the PVN is considered as an initial regulator for the activation of the hypothalamo–pituitary–adrenal (HPA) axis (Pacak et al. 1995). Hashimoto et al. (1993) reported that low plasma CRH levels in patients are associated with non-insulin dependent diabetes mellitus (NIDDM).

Arginine vasopressin (AVP) is a nine amino acid/nonapeptide neurohormone and exhibits three main physiological roles: regulation of water homeostasis, vascular constriction, and control of ACTH secretion (reviewed in Murat et al. 2012). Several investigators have reported that the expression of AVP in parvocellular neurons of the PVN and V1b receptor density in pituitary corticotrophs is significantly increased in response to chronic stress (Sawchenko, 1987; Kovacs and Sawchenko, 1996; Aguilera and Rabdan-Diehl, 2000a, b). It has been also shown that diabetes mellitus is associated with hyperosmotic dehydration in which AVP level was decreased (Young, 1969).

Although a lot of studies showed the relation between CRH and AVP with different types of stress (Yadawa and Chaturvedi, 2016) but the direct correlation of diabetes and activation of HPA axis with reference to CRH and AVP is not yet known. Our results showed a significant increase in the immunoreactivities of
hypothalamic CRH and AVP as well as increase in the number of neurons in the PVN region of both STZ and ALX induced diabetic groups compared to control (Fig.I-10 and Fig.I-11). Moreover, serum corticosterone also increased significantly in both the diabetic groups compared to control (Fig.I-12). The elevated level of corticosterone may be due to synergistic effect of CRH and AVP which act upon pituitary corticotrophs to release adreno-corticotropin releasing hormone (ACTH). This ACTH acts upon adrenal glands to secrete corticosterone, the major stress hormone produced from the cortex of the adrenal gland which plays a regulatory role in stress induced HPA axis activity in rodents (Osterlund and Spencer, 2011).

Similar results were found in avian species like Japanese quail and chickens where AVT (avian isoform of AVP) expression was upregulated in water deprived conditions (Chaturvedi et al. 1994, Chaturvedi et al. 2000, Seth et al. 2004). Results from present study have demonstrated that in mice, chronic hyperglycemia is not only associated with increased immunoreactivity of hypothalamic CRH and AVP but also recruits many more neurons to increase the rate of CRH and AVP synthesis and secretion in the PVN. Present findings suggest that the increased expression of CRH and AVP in the PVN in combination activate pituitary ACTH and hence may contribute to the activation of HPA axis as a measure during stressful diabetic condition.

Taking into account various parameters observed in the present study, it is obvious that both the diabetogens affect islet architecture and function as well, especially the expression of ir-insulin and Kir6.2. However, in general, the degree of effect in modulating these parameters is higher in STZ treated mice compared to ALX in both brain and pancreatic tissues. But antioxidant activity of brain catalase enzyme differs markedly in the two diabetogens. It increases significantly in STZ treated mice
while decreases in ALX group compare to control. On the other hand, pancreatic catalase although decreases in both the diabetogen group, the decrease was 3 fold in STZ vs 2 fold in ALX group compare to control (Fig.I-4). This comparison indicates that STZ is more potent diabetogen as well as induces greater degree of effect in terms of oxidative and nitrosative stress.

Further, STZ appears to affect HPA axis more potently specially the feedback effect of corticosterone level. Although serum corticosterone level and ir-CRH + AVP increased in both STZ and ALX treated groups compared to their respective controls, it is apparent that higher the level of corticosterone (STZ > ALX) lesser was the increase in the expression of ir-peptides CRH and AVP (STZ< ALX) (see Fig.I-10, I-11 and I-12). Hence it is worth mentioning that after inducing experimental diabetes via these drugs, degree of effect is more in STZ compared to ALX treated mice. For example, degree of decrease of ir- insulin and Kir6.2 was more in STZ than in ALX treated mice (STZ > ALX) and moreover, corticosterone level was higher in STZ compared to ALX group.

In terms of oxidative stress or the modulation of antioxidant enzymes, in general, effects of greater degree were also observed in STZ treated mice compare to that of ALX. It is also suggested that STZ is more active diabetogen causing long term complications since blood glucose level remains at a plateau after attaining the peak level while in ALX it starts decreasing after attaining the peak (Fig. I.1B). Moreover, it also appears that STZ utilizes different mechanism at the brain level since catalase activity in the brain increases but decreases in the pancreas of STZ treated mice (compare to control) while in case of ALX, activity of this antioxidant enzyme decreases in both the tissues compare to control (Fig. I-3 and I-4). Further,
other antioxidant enzymes (SOD and GPx) followed the same pattern in brain and pancreas as well as in both STZ and ALX groups.

Since it appears that amount/level of oxidative and nitrosative stress is higher in STZ treated mice, the interrelation and/or interdependence of DM and free radical species cannot be ruled out. It is concluded that degree of diabetic complications depends on the onset/intensity of diabetes and certain specific parameters (especially oxidative and nitrosative stress) may be used as an indicator of pre-diabetes as well as diabetic complications. Although further studies are required to assess the molecular basis of diabetic complications at the level of different organs such as brain (neuropathy), retina (retinopathy) and kidney (nephropathy) causing multiple complications.

In summary, the comparative study of STZ and ALX induced diabetes mellitus indicates that both the diabetogens affect islet function/architecture as well as activate the stress/HPA axis, but the degree of effect is significantly greater in STZ treated mice in comparison to that of ALX. Further, hyperglycemia is invariably associated with oxidative and nitrosative stress which in turn accelerates diabetic complications and the activation of hypothalamic-pituitary-adrenal axis.

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Table I-1  A comparative study of STZ and ALX treatment on various parameters. Values are presented as mean ± S.E.M (n = 5).

*P<0.05 **p< 0.01, ***p<0.001; significance of difference from control.
#<0.05 ##p< 0.01, ###p<0.001; significance of difference from STZ.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CONTROL</th>
<th>STZ</th>
<th>ALX</th>
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<tbody>
<tr>
<td><strong>BRAIN</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NOx (μM/mg of protein)</td>
<td>10.59±1.04</td>
<td>27.64±0.35**</td>
<td>23.53±1.03###</td>
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<td>CAT (U/mg of protein)</td>
<td>0.891±0.0</td>
<td>1.589±0.0**</td>
<td>0.682±0.0####</td>
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<td>GPx (U/mg of protein)</td>
<td>0.0098±0.0</td>
<td>0.0176±0.0**</td>
<td>0.0010±0.0###</td>
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<tr>
<td>SOD (U/mg of protein)</td>
<td>0.0182±0.00</td>
<td>0.0955±0.00**</td>
<td>0.0316±0.01####</td>
</tr>
<tr>
<td>MDA (nM/mg of protein)</td>
<td>11.02±5.5</td>
<td>21.82±10.9**</td>
<td>32.69±16.3####</td>
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<td>AR (U/mg of protein)</td>
<td>8.251±0.75</td>
<td>12.83±0.50*</td>
<td>14.64±1.64###</td>
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<tr>
<td>AGEs (AU/mg of protein)</td>
<td>2.64±0.11</td>
<td>3.38±0.17*</td>
<td>3.83±0.03*</td>
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<tr>
<td>AOPP (μM/mg of protein)</td>
<td>620.4±15.77</td>
<td>1088.2±13.98**</td>
<td>808.1±17.08###</td>
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<tr>
<td>N/L ratio</td>
<td>0.097±0.015</td>
<td>0.403±0.048**</td>
<td>0.353±0.071###</td>
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<td>CORT (ng/ml)</td>
<td>483.33±13.33</td>
<td>2466.6±33.5***</td>
<td>1650±27.3####</td>
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<td><strong>PANCREAS</strong></td>
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<td>NOx (μM/mg of protein)</td>
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<td>19.48±0.34**</td>
<td>16.58±0.25###</td>
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<td>1.038±0.13###</td>
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<td>SOD (U/mg of protein)</td>
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<td>AOPP (μM/mg of protein)</td>
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<td>915.12±28.96###</td>
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