CHAPTER 5

DISCUSSION
Brain is extremely sensitive to disturbance in CBF and if this persists for long time, neuronal cells are at the thresholds of damage. To accomplish such interruptions, brain receives effective perfusion through finely distributed blood vessels and their collaterals, which manages the high metabolic demands. However, during cerebral stroke, interruption of blood supply activates several multifaceted and overlapping mechanisms, which modulate the survival and death of brain cells (Mehta et al., 2007; Nakka et al., 2007). Further, besides the duration of ischemia being a critical determinant of subsequent brain damage, the reperfusion does also trigger signaling pathways deleterious to cells.

Cerebral stroke has always been associated with various risk factors such as diabetes, which increases the stroke incidence by at least 4-12 times with severe impact on outcomes leading to severe morbidity and mortality. Further, besides being a deadly disorder, with reduced survival in diabetics, stroke itself is a long term disabling condition, as most stroke survivors are left with some sensori-motor and/or cognitive deficits and once damage from stroke has matured, little can be done to recover the impaired/lost function. Moreover, the association between diabetes and consequent outcome following stroke has largely been usually reported with death as opposed to disability as a primary outcome in normoglycemic subjects (Oppenheimer et al., 1985; Scott et al., 1999; Gray et al., 2007). Further, it has been suggested that cerebral ischemia simultaneously evoke cellular survival mechanisms and their competition with cell death regulatory molecules ultimately decide the fate of cells. However, the mechanisms by which diabetes aggravates ischemic brain damage are still a subject of detailed study. Therefore, the present study was undertaken to elucidate the cellular and molecular mechanisms involved in exaggerating the ischemic cerebral damage in diabetes. Moreover, the major emphasis was given to discern the role of cell death regulatory molecules such as cytochrome c, Bax, caspase-3, AIF and PARP, although various molecules, such as HSP70, Bcl-2, involved in cell survival is also studied to understand the basis of cerebral ischemia induced brain damage in diabetes.

It was observed that transient focal cerebral ischemia/reperfusion induced by reversible unilateral MCAo in STZ-diabetic rats, impaired the brain function, which was clearly marked by altered sensori-motor functions. The MCAo by intraluminal filament occlusion technique used presently is the most widely used model for inducing ischemia. The placement of the suture at the origin of the MCA obstructs blood supply to the total MCA supplying territory including the basal ganglia, which are supplied by
the lenticulo-striate arteries. These MCA branches are end-arteries, which in contrast to the cortical branches do not form collaterals. Therefore, impaired blood supply to the MCA territory as consequences of focal ischemia was evaluated using a simple, rapid behavioral examination with quantitative grades for each symptom. It was found that increase in I/R, significantly increased the neurological deficit in diabetic rats as revealed by varying degrees of flexion, contralateral circling, hemiparesis and non-spontaneity. This is in agreement with other reports suggesting that cerebral ischemia induced greater damage in diabetics with enhanced neurological deficit than nondiabetic animals. All of these deficits can probably be attributed to brain lesions affecting sensori-motor areas such as localized in the striatum, primary motor cortex, the anterior dorsal cortex areas corresponding to the hind limb and the forepaw. These cortical and striatal structures are involved in the processing of sensori-motor information (Andersen et al., 1991; Scremin, 1995). However, it is often difficult to distinguish the extent to which neuronal damage in each of these two brain regions contributed to altered sensori-motor performance, because the caudate putamen receives extensive input from the sensori-motor areas of the cortex. Neurotoxic and or electrolytic lesioning of the dorsomedial striatum tends to have generalized effects on the locomotor activity, whereas neuronal damage that extended into the caudate putamen affected the sensorimotor orientation and skilled motor control (Carli et al., 1985; Sabol et al., 1985). However, in preischemic hyperglycemia or diabetes, the damage surprisingly occurs much faster. This was clearly supported by morphometric analysis of TTC stained brain slices, which revealed increased infarct size in diabetic as compared to normoglycemic rats at respective time points of I/R injury.

Further, it was found that ischemic brain damage at early time point of I/R injury was largely confined to the striatum but it extends to the cortical region in diabetic animals upon increasing the ischemia or reperfusion time (Du et al., 1996; Nedergaard, 1987; Li et al., 1998; Gisselsson et al., 1999). The progressive increase in ND score (>8) and cerebral damage upon extending the I/R time (2/24 h) resulted in increased mortality in diabetics. This implies that increased I/R injury intensify the stress in diabetes and which was later responsible for progression and maturation of damage and ultimately greater mortality in diabetics with increasing reperfusion time.

These results were further strengthened by histological findings with H&E staining, which revealed cellular damage characterized by necrotic, apoptotic and both type of features. The extent of cellular damage very well correlated with the severity of
ischemic stress, which was greater in diabetics. The striatum appeared to be the most affected brain region following I/R of 1/24 h as compared to 0.5/24 h of I/R in diabetics and showed cavitations and cell loss. The cellular damage in this region of the brain is predominantly necrotic in nature, which was greater in diabetics as compared to normoglycemic ones. However, the end margins of striatum in diabetics at 1/24 h of I/R showed some sign of cellular damage characterized by apoptotic morphology. In contrast, the cortical area of 0.5/24 h of I/R in normoglycemic appeared to be normal, however, the diabetic group at respective time point showed clear sign of cellular damage. Further, when the I/R time increased to 1/24 h, the damage also increased progressively, which was conspicuously greater in diabetics. The cellular alterations appeared to be more of apoptotic nature in this brain loci and which are markedly much higher in diabetics. The hippocampus showed normal cellular morphology at both these time points. Further, the concomitant activation of both forms of cell death in same cell is consistent with earlier reports (Unal-Cevik et al., 2004). Additionally, the predominant cell death phenotypes in these brain loci depends on duration and severity of ischemic insult and are determined by the relative speed of each process, which seems to be enhanced in diabetes. As mentioned above the striatum being greatly affected by ischemic insult and displayed DNA fragmentation of type I TUNEL-positive cells, lacking characteristic morphological features of apoptosis; such as cell shrinkage, nuclear condensation, or nuclear fragmentation, however, end margins of striatum also showed cells exhibiting characteristic features of apoptotic cell death. The large number of neurons surrounding the infarct in cortical area exhibited prominent type II TUNEL positive staining, a telltale of apoptosis. The increased number of TUNEL positive cells at 1/24 h of I/R is about 44 and 70% in striatum of normoglycemic and diabetic rats respectively as compared to cortex, being about 13 and 57%. This indicates the staining of necrotic cells due to extensively damaged DNA. However, staining with Hoechst clearly revealed the apoptotic morphology of their nuclei, which is quite different to that of primary necrotic cells. TUNEL-positive cells observed 24 h post reperfusion injury may be one of the mechanisms important in the maturation of a cerebral infarct and responsible for underlying protracted loss of neuron. Further, as a consequence of the striatal region consistently suffer severe reduction of blood flow, whereas the cerebral cortex exhibits a gradient of decrease in blood flow from the peripheral towards the central parts of the vascular territory. Therefore, the cell death in these two very brain regions was obiously dependent on
the availability of energy resulting into the DNA fragmentation of necrotic and apoptotic nature. However, virtually absence of TUNEL positive cells in the hippocampal region may be due to its anatomical location, which is relatively out of reach to MCAo. Few TUNEL positive cells (∼1 and 5 %) observed in the hippocampal region of normoglycemic and diabetic rats respectively may be due to secondary damage and may be perceived as normal physiological event. Therefore, the type of cells undergoing necrotic/apoptotic damage may represent the differential sensitivity of ischemic insult to various brain nuclei.

The increased cerebral damage from 0.5/24 to 2/24 h of I/R injury in diabetics may be due to free radical generation, since, we found significant increase in lipid peroxidation by-product MDA in serum in diabetics of 0.5/24 ($P<0.01$) and 1.24 h ($P<0.05$) in comparison to normoglycemic rats. Further, the MDA levels at 2/24 h of I/R in normoglycemic and diabetic rats were nearly similar, which may be due to extensive cerebral damage, which may have prevented the MDA release in blood. Nevertheless, the increased level of MDA reflects the severity of oxidative stress due to generation of ROS as a consequence of focal cerebral ischemia and in particular reperfusion injury. The presence of MDA in blood may be due to the BBB damage (Belayev et al., 1996; Albayrak et al., 1997). The reactive oxygen free radicals are known to cause lipid peroxidation and membrane damage. The increased TBARS level paralleled with the ND scores and were further supported by overall decrease in GSH level. The continuous decrease in GSH level in diabetic and normoglycemic animals indicates the predominance of oxidative stress. Further, the loss of scavenging systems and nonreplenishment of GSH content following cerebral ischemia in diabetics appears to enhance the cerebral damage. Brain in particular is very susceptible to oxidative stress owing to its high lipid content, high oxidative metabolic activity and low antioxidant defense. Therefore, increase in lipid peroxidation (MDA) can result from an overproduction of free radicals and partly may be due to severe GSH depletion in diabetics.

Above results clearly indicate the activation of cell death pathway(s), which were responsible for increased damage in diabetes. One of the reasons for activation of cell death mechanism is oxidative stress as indicated by higher MDA and reduced GSH level in diabetics. Moreover, in the present study, the cellular changes at 0.5/24 h of I/R in normoglycemic was not significant but increase in the time points of I/R to 2/24 h resulted in high mortality (>90%) in diabetics. Which led us to carry out systematically
the detailed investigation for biochemical, cellular, and molecular basis of diabetes induced increased cellular stroke damage at 1 h of ischemia and 3, 6, 12 and 24 h of reperfusion injury.

The cellular and DNA fragmentation analysis revealed at 1/24 of I/R is suggestive of late event in apoptotic cell death and seems to be responsible for maturation of cellular damage in diabetic rats. One of the earlier stages of apoptosis is the loss of plasma membrane symmetry by translocation of PS to the external milieu upon flipping from inner leaflet of the plasma membrane to outer leaflet (Denecker et al., 2000). This early event in apoptosis allows the recognition of apoptotic cells to phagocytic cells in order to eliminate damaged cell without cell proteolytic enzyme leakage (Fadok et al., 1992). Therefore, we further confirmed and investigated the initiation of early apoptotic events by using Annexin V assays. Interestingly, it was found that cerebral ischemia induces significantly \( P<0.01 \) increased early (1/3 h of I/R) initiation of apoptosis in both cortical (45 and 126 %) and striatal area (66 and 124%) of normoglycemic and diabetic rats respectively. The apoptotic events significantly increased further (155 %) at 1/24 h \( P<0.01 \) in cortical area of diabetics in comparison to normoglycemic (~70 %), which may be due to the maintenance of cellular energy level in this affected region of the brain. However, the decrease in apoptotic changes in striatal area at 1/24 h in diabetic (55 %) and normoglycemic (30 %) animals suggests that striatal area is severely damaged as early as 1/3 h of I/R injury due to metabolic derangements. Moreover, the cell death comprised by mixed characteristic of apoptosis and necrosis, termed necroptosis, which may be due to switch from apoptotic to secondary necrosis. Further, the increased cavitations and also cell loss observed with H&E staining in striatal area indicates the clearance of apoptotic/necrotic cell by phagocytic cells. These two affected regions, striatum and cortex, of the brain seems to form the core and penumbra respectively, since, it was suggested according to the threshold concept of ischemia that structural integrity of the brain is preserved in the penumbral area, which is characterized by a loss of function, and the ischemic core, in which both functional and structural integrity are severed. These two brain regions were further demarcated for core and penumbra using molecular stress marker HSP70, since it has been suggested that HSP70 induction was highest, where perfusion reduction was less severe and HSP70 was induced mostly in the periphery and not in the core region (Weinstein et al., 2004), quite supportive of our results.
The predominance of oxidative stress, which might have resulted into increased brain damage in diabetics as revealed by MDA level, was further confirmed by the ROS level in the affected brain nuclei of normoglycemic and diabetic rats. The oxidative stress appeared to have direct impact on brain damage following cerebral ischemia in diabetes. In the present study, increased ROS generation has been observed as early as 3 h post reperfusion in both striatal (~30 and 50 %) and cortical area (~20 and 27 %) of normoglycemic and diabetic rats respectively. Increased cellular production of ROS occurs primarily in the mitochondria through imperfectly coupled electron transport system and oxidative stress has been widely accepted as key mediator in the development and progression of diabetes and its complications due to increased production of free radicals and impaired antioxidant defenses (Evans et al., 2003; Ceriello, 2003; Maritim et al., 2003). This can lead to a self perpetuating process, since radicals product itself are both reaction initiators as well as the products of lipid peroxidation. Lipid peroxy radicals, such as MDA, react with other lipids, proteins, and nucleic acids, thereby propagating the transfer of electrons and bringing about the oxidation of substrates. The polyunsaturated fatty acid (PUFA) present in the membranes, are highly susceptible to oxidative attack and, consequently, changes in membrane fluidity, permeability and cellular metabolic functions. Further, ROS generation is suggested to be a causal link between high glucose, mitochondrial dysfunction and apoptosis (Russell et al., 2002). Increased ROS generation and poor antioxidant defense also lead to deregulation of cellular processes, and mutations of the genome, thereby causes aberrations in ion homeostasis, cell signaling and gene expression by redox regulation of transcriptional factors/activator and/or by oxidatively modulating the protein kinase cascades and induce various early response or stress-response genes like c-fos, c-jun, jun-B, jun-D etc (Matsuzawa and Ichijo, 2005). The involvement of ROS in DNA damage was supported by significant \( P<0.001 \) reduction in TUNEL positive cells from about 44 and 70 % to 11 and 27% in the affected brain territory of normoglycemic and diabetic rats respectively following post reperfusion treatment with melatonin.

However, it is evident that mitochondria play a central role in both apoptotic and necrotic cell death (Kroemer et al., 2007), alterations in the mitochondrial potential \( \Delta \Psi_m \) observed in the present study led to an early release of mitochondrial proteins such as cytochrome c and AIF. Therefore, diabetes seems to generate ROS coupled with mitochondrial membrane hyperpolarization (MHP) followed by mitochondrial
membrane depolarization (MMD). The $\Delta \Psi_m$ hyperpolarization followed by MMD due to high glucose generated ROS is suggested to be temporally related to an increase in ADP:ATP ratios and an absolute decrease in ATP levels. This in turn is coupled with cytochrome c release from the intermitochondrial membrane space and cleavage of caspase-3 resulting in apoptosis (Russell et al., 2002). Further, the early increase in $\Delta \Psi_m$ as compared to depolarization, may be a physiologically more relevant step in inducing apoptosis (Krohn et al., 1999) and that cytochrome c is actively released in response to MHP, while cytochrome c release after depolarization is suggested to be merely a passive response to mitochondrial swelling (Poppe et al., 2001) or related to permeability transition pore regulated by Bax and Bcl-2 proteins (Green and Reed, 1998). A frequent, but not invariable, requirement for translocation of cytochrome c from the intermitochondrial space to the cytosol is either MHP or MMD (Green and Reed, 1998). These results confirm the previous reports demonstrating direction of mitochondrial membrane polarization, which depends on the extent of oxygen glucose deprivation (OGD), as long OGD results in depolarization, while shorter OGD induces hyperpolarization (Iijima et al., 2003a,b). MHP has been reported as a pathological step in various experimental settings induced by the excessive excretion of protons or inhibition of proton reentry due to limitation of reentry of protons through FoF1-ATPase upon inadequate supply of ADP (Kim et al., 2003; Rubi et al., 2004; Perl et al., 2004; McLeod et al., 2004). Such disturbance of FoF1-ATPase and adenine nucleotide translocase has been earlier reported under postischemic condition by Di Lisa and Bernardi (1998). Thus, hyperpolarization during reperfusion implicates the pathological mechanism on post-ischemic neuron. The mitochondrial hyperpolarization precedes neuronal death and neurons remain viable during hyperpolarization but the process may switch on the apoptotic cascade (Iijima et al., 2006). Mitochondrial dysfunction may be subject of increased oxidative stress as melatonin, a well known antioxidant, seems to normalize the $\Delta \Psi_m$ (Han et al., 2006) and reduce the ROS level. Additionally, the early dynamic change of mitochondrial morphology is a causal factor necessary for hyperglycemia induced ROS increase (Yu et al., 2006). It is suggested that upregulation of glucose transporters as in diabetes by NO might be detrimental in conditions characterized by excessive glucose supply (Mastrocola et al., 2005). Moreover, mitochondrial hyperpolarization in hyperglycemic condition is due to increase input of metabolic substrate into mitochondria overwhelming the electron transport system, which results into overproduction of ROS (Brownlee, 2001;
Nishikawa et al., 2000; Du et al., 2001; Lin et al., 2005). However, the impact of ΔΨm to the release of proteins needs further investigations; nevertheless, it provides a direct evidence of mitochondrial dysfunction and ROS generation.

Additionally, as discussed above, mitochondrial permeabilization may also be mediated by Bax, which is translocated from cytosol to mitochondria in the present study as early as 3 h (~90 %) post reperfusion in diabetic as compared to normoglycemic animals (~50 %). The increased translocation of Bax to mitochondria in cortical area supports apoptotic mechanisms as a major cause of cell death in this very brain locus. However, the early decrease in Bax in striatal region in diabetic as compared to normoglycemic rats suggests the necrotic cell death prominence, although increase at later time points may be at the end margins or those survived the initial shock.

Moreover, the changes in the mitochondrial membrane permeability have been suggested to be regulated by antiapoptotic Bcl-2 protein. Which thereby inhibit apoptosis and improves neuronal survival following cerebral ischemia. Therefore, we investigated the expression of prosurvival Bcl-2 protein. It was found that Bcl-2 expression significantly increased at 1/3 (P<0.05) and 1/24 h (P<0.01) of I/R in cortex of diabetics. Therefore, it appeared that these two time points are crucial for the initiation and maturation of cell death as revealed by increased early apoptotic changes with Annexin V staining. However, the increased expression of Bcl-2 in the cortical region may not be sufficient to inhibit the ongoing process, but appeared to have different role by maintaining the mitochondrial membrane integrity, preventing lipid peroxidation and scavenging free radicals (Kane et al., 1993). Further, there was no decrease in the Bax levels inspite of the overexpression of Bcl-2 in the cortical region and this is in agreement with earlier report that Bcl-2 overexpression does not necessarily alter Bax expression (Zhao et al., 2003). In contrast, the significant decrease in Bcl-2 expression at 1/3 (P<0.01) and 1/24 h (P<0.001) in striatal area of diabetic is in agreement with the rapid cell loss due to necrosis and suggests down regulation of survival pathways. Thus, higher level of ROS in striatum of diabetic rats at 1/3 h of I/R in comparison to cortex at respective time point indicates the preservation of cellular integrity in cortex.

The early release of cytochrome c from mitochondria observed in the present study in diabetic animals is the central component of the platform through which caspase cascade is initiated by activation of family of caspases particularly caspase-3 (Muranyi
et al., 2003). The release of cytochrome c in cortical area coincides with the Bax translocation, whereas in striatal area, the initial increase seems to be dependent on loss of membrane integrity and at later time due to Bax dependent membrane permeabilization in survived cells.

One of the factors in induction of caspase cascade is availability of ATP, since increased ATP supply favours the apoptotic mode of cell death, and decreased ATP favours the necrotic type of cell death (Eguchi et al., 1997; Leist et al., 1997). It is suggested that, in focal cerebral ischemia, the brain energetic state is better preserved in diabetic than in normoglycemic animals following recirculation (Hillered et al., 1985; Folbergrova et al. 1992). Therefore, the greater activation of caspase-3 as reported presently following I/R injury under diabetic conditions is the direct indicator of activation of apoptotic machinery. The significantly increased caspase-3 mRNA in the cortical area of diabetic rats further substantiates these results. Caspase-3 is considered to be the executioner of apoptotic cell death, since; it activates calpains, caspase-activated endonuclease CAD/DFF40 (Stennicke and Salvesen, 1998; MacManus and Buchan, 2000), cleave PARP and thereby ensues breakdown of nuclear structure and bioenergetic metabolism (Eliasson et al., 1997; Krupinski et al., 2000).

Our results indicate that PARP is cleaved in diabetics as early as 3 h post reperfusion, which further support caspase-3 activation. Moreover, a significant increase in apoptosis as indexed by TUNEL and caspase-3 localization at 24 h post reperfusion in cells with apoptotic morphology following the induction of focal ischemia in the present study further confirms our findings. This suggest that mitochondrial dysfunction and mitochondria-induced cell death pathway, which involves cytochrome c release and caspase-3 activation, may play a key role in pathogenesis of diabetes-enhanced ischemic brain damage.

Although caspase-mediated apoptosis is the main form of cell death, however, accumulating evidence now also show that caspase-independent pathways are equally important (Leist and Jaattela, 2001; Li et al., 2001). Further, some cells found in the present study showed features typical of caspase-3 independent apoptosis such as peripheral chromatin condensation on the nuclear membrane indicating concomitant activation of caspase dependent and independent cell death mechanisms. One of the reasons for this seems to effectively eliminate the nonrepairable and irreversibly damaged cells. It was observed in the present study that in addition to cytochrome c, AIF was also released from the mitochondria (Susin et al., 1999). Increased
translocation of AIF to cytosol and nucleus in diabetes as early as 3 h post reperfusion, indicates its involvement in cell death mechanisms. Thus, the present results are in agreement with the earlier report demonstrating AIF relocation from mitochondria to nucleus in primary cultured rat neurons 4 and 8 hours after 4 hours of oxygen/glucose deprivation and also in ischemic mouse brain, after 45 minutes MCAo and 1 h reperfusion. AIF translocation preceded cell death, occurred before or at the time when cytochrome c was released from mitochondria, and was evident within cells showing apoptosis-related DNA fragmentation (Plesnila et al., 2004). The signal for AIF release seems to be initiated by PARP, in molecular ordering of PARP, calpains, Bax, and AIF (Moubarak et al., 2007), whose expression increased markedly at 3 h post reperfusion, suggesting the involvement of PARP in conversation between nucleus and mitochondria (Yu et al., 2006). It has been reported that AIF translocation persists following ATP depletion, suggesting that AIF still works in conditions, where caspase-3 fails and the presence of pan-caspase inhibitor Z-VAD.fmk could not prevent the mitochondrio-nuclear translocation of AIF (Daugas et al., 2000). Therefore, AIF appears to play a key role in programmed cell death in striatal region, which otherwise die by necrosis due to lack of energy, although caspase-3 mediated cleavage of PARP in this region seems to be in cells, which are still viable but stressed. The release of AIF in striatum may depend upon the pore formed by Bax or mitochondrial dysfunction at early hours of reperfusion, whereas, it seems to be released at later time points of reperfusion through breakdown of mitochondrial integrity. In contrast AIF and cytochrome c release seems to be Bax dependent at later time point of I/R in cortex.

It is suggested that AIF propagate the first wave of apoptosis, indispensable for early embryonic morphogenesis (Joza et al., 2001) and expression of AIF in the normal rat brain decreases slightly, whereas caspase-3 exhibits dramatic decrease paralleling with Apaf-1 during normal development (Zhu et al., 2005). Further, AIF displayed a more pronounced translocation in neurons of neonatal male mice after hypoxia-ischemia, but the female brain neurons displayed a stronger activation of caspase-3, making neurons as an appropriate AIF-target during stroke (Zhu et al., 2006). The AIF release is dependent on its cleavage near N-terminus by calpain I and the truncated AIF was reported to be essential for its translocation (Cao et al., 2007). The lethal translocation of AIF to the nucleus requires interaction with cyclophilin A to cause peripheral chromatin condensation and large-scale (50 kb) DNA fragmentation (Daugas et al., 2000; Zhu et al., 2007). The AIF deficiency in mitochondria may also
compromises mitochondrial oxidative phosphorylation and seems protective in diabetes (El Ghouzzi et al., 2007; Pospisilik et al., 2007) thereby signifying its importance and location in cell physiology. The transcriptional regulation of AIF seems strictly dependent on the function of AIF in different cellular compartments presently. Thus, AIF mRNA down regulation at later time points of I/R in diabetic rats indicates the inhibition of protective ROS scavenging role of AIF in irreversibly damaged cell. However, it has been suggested that AIF is antagonized and sequestered by HSP70 following hypoxic/ischemic injury (Ravagnan et al., 2001; Matsumori et al., 2005) and heat shock or stress proteins represents an emerging paradigm for the coordinated, multistep regulation of apoptotic signaling events to provide protection from and to facilitate cellular recovery after exposure to damaging stimuli both in vitro (Rordorf et al., 1991; Papadopoulos et al., 1996; Kelly et al., 2001; Lee et al., 2001) and in vivo (Rajdev et al., 2000; Hoehn et al., 2001; Tsuchiya et al, 2003). The mechanism of neuroprotection may be attributed to the function of HSP70 as a molecular chaperone protein that antagonizes apoptosis in both caspase-dependent and -independent pathways. Additionally, HSP70 also acts as a molecular stress signal and its altered expression following I/R suggests the increased sensitivity and distribution of damage in diabetic brain. However, we have observed the decreased expression of HSP70 in striatum of diabetics, which is in direct correlation with the increased translocation of AIF at respective time point. The striatal region, which forms the core region due to diminution of the HSP70 expression, may be due to decrease in cerebral protein synthesis during ischemia (Kokubo et al., 2003). The cortex on the other hand showed increased expression of HSP70 in diabetics at 1/24 h of I/R, could be due to specific induction as penumbral cells are reversibly injured and may survive ischemic shock (Muranyi et al., 2005). The HSP70 expression at mRNA level was nearly similar in both diabetic and normoglycemic animals suggesting that the cells, which are destined to die in severely ischemic areas of the brain may transcribe HSP70 mRNA but may not translate it into HSP70 (Nowak and Jacewicz, 1994; Kinouchi et al; 1993). Moreover, HSP70 stress protein induction represents an endogenous protective mechanism that occurs in penumbra but not in core area (Weinstein et al., 2004) Further, it is not clear that HSP70 interact with AIF at these time points, nevertheless HSP70 acts as a marker to distinguish ischemic core and penumbra (Weinstein et al., 2004, Sharp et al., 2000).
Therefore, taking into consideration that the reperfusion phase after ischemia is responsible for enhanced cerebral damage in diabetes, we choose to examine the effect of melatonin on various parameters in MCAo model with 1 h occlusion followed by 24 h reperfusion. We followed the cascade of events that involves increased ROS generation, mitochondrial dysfunction and activation of downstream cell death pathways leading to subsequent DNA fragmentation. Our results clearly demonstrated attenuation of ROS generation, normalization of mitochondrial potential and reduction in DNA damage. These effects of melatonin are in agreement with other workers, which showed that melatonin inhibits apoptosis in ischemic kidney (Kunduzova et al., 2003), also in amyloid beta-peptide injury in hippocampal neurons (Shen et al., 2002) and NO-induced cell death in PGT-beta immortalized pineal cells (Yoo et al., 2002). The antiapoptotic effects of melatonin is mediated in conditions involving mitochondrial dysfunction (Andrabi et al., 2004), strongly support our results, since melatonin does not protect against staurosporine-induced apoptosis, which is known to follow pathways that do not involve mitochondrial depolarization, leading to the opening of mitochondrial permeability transition pore (Krohn et al., 1999; Harms et al., 2000). Further, melatonin can strongly prevent the OGD-induced loss of the mitochondria membrane potential; suggests that the direct inhibition of mitochondrial pathway might essentially contribute to its antiapoptotic effects in neuronal ischemia-reperfusion (Han et al., 2006). The neuroprotective effect of melatonin is thus clearly reflected by reduced infarct size and improved neurological function, when administered at the onset and 6 h post-reperfusion. However, we have observed that single injection of melatonin at 30 min prior to ischemia or at onset of reperfusion did not seem to significantly reduce cerebral infarct size. Thus, it seems that 3-6 h reperfusion further initiate cellular damage processes in the brain and therapeutic intervention by melatonin seems to provide beneficial clinical application for treating stroke and other neurodegenerative disorders.

The results of the present study therefore clearly highlights the activation of mitochondria-mediated pathway(s) by caspase-3 dependent and independent apoptotic mechanisms, in enhancing ischemic cerebral damage in diabetes. This may partly be due to enhanced ROS generation leading to oxidative stress since, a well known antioxidant, melatonin reduced ROS level, normalized mitochondrial potential, reduced DNA damage and infarct size and tended to improve neurological function as well.