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Ischemic Stroke in Type II Diabetic Mice: Deregulation of SDF-1a/ CXCR4 Axis

Avik Das
Wright State University

ISCHEMIC STROKE IN TYPE II DIABETIC MICE:

DEREGULATION OF SDF-1a/CXCR4 AXIS

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By

AVIK DAS
B.Pharma., Berhampur University, Orissa, India, 2005

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Wright State University

WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY AVIK DAS ENTITLED "Ischemic Stroke In Type II Diabetic Mice: Deregulation of SDF-1a/CXCR4 Pathway," BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

_____

Yanfang Chen, M.D. Ph.D.
Thesis Director

_____

Mariana Morris, Ph.D.
Department Chair

Committee on Final Examination:

_____

Yanfang Chen, M.D. Ph.D.

_____

Khalid Elased, Ph.D.

_____

Mariana Morris, Ph.D.

_____

Joseph F. Thomas Jr., Ph.D.
Dean, School of Graduate Studies

ABSTRACT

Das Avik. M.S., Department of Pharmacology & Toxicology, Wright State University, 2009.
Ischemic Stroke in Type 2 Diabetic Mice: Deregulation of SDF-1a/CXCR4 axis[1].

[1]Type 2 diabetes mellitus is a major risk factor for ischemic stroke. Also diabetes is associated with poor outcome after stroke. Underlying mechanisms are however not fully understood. Alteration in the expression of the SDF-1a/CXCR4 axis, which is important for ischemic tissue repair, can be a probable cause. In this study, we have determined the expression of SDF-1a/CXCR4 in the brains of type II diabetic mice at basal and in response to ischemic stroke and have investigated a method for overexpression of SDF-1a in the brains of the diabetic mice. Adult male C57BLKS/J mice (db/db) of age 8 weeks were used as the murine model for type II diabetes and their age matched lean littermates served as controls (db/+). Microvascular density was first determined in the cerebral cortex of db/db diabetic mice by immunohistochemical analysis. Focal cerebral ischemia was induced by middle cerebral artery occlusion surgery (MCAO) in type 2 diabetic db/db mice and their controls. 48 hours after surgery, volume of ischemic damage was determined by TTC staining. The expression of SDF-1a and CXCR4 in the ischemic and non ischemic sides of brains of both the groups were determined using western blot and real time RT PCR. The db/db diabetic mice were injected with the

vector, adeno associated virus 9 (AAV-SDF-1a) in the brain striatum and the overexpression of SDF-1a was determined by immunohistochemical analysis. Double immunohistochemistry was used to determine the localization of SDF-1a in brain after injection of the vector. The microvascular density in the cerebral cortex was reduced in db/db mice as compared with db/+ mice ($p<0.05$). Volume of ischemic damage was significantly increased in db/db mice after focal cerebral ischemia ($p< 0.01$). The levels of SDF-1a expression in both ischemic and non ischemic side of brain were reduced in db/db mice as compared with those in db/+ mice at mRNA ($p< 0.01$) and protein level ($p< 0.01$). The amount of CXCR4 expression was significantly reduced only in the ischemic side of the brains of db/db mice at protein level ($p=0.001$) and at m-RNA level ($p=0.001$). But in the non ischemic side, the expression of CXCR4 did not show any significant difference between the two groups. Immunohistochemical analysis showed overexpression of SDF-1a in the striatum receiving the microinjection of AAV-SDF-1a and double immunohistochemistry showed SDF-1a to be localized in the glial cells of the cerebral striatum after microinjection. The results indicate that microvascular density is reduced and ischemia induced cerebral damage is enlarged in diabetes which may be linked to the impaired expression of hypoxia regulated SDF-1a/CXCR4 axis after ischemic stroke in diabetes and vector mediated over expression of SDF-1a in the brain can be a novel therapeutic technique for treating ischemic stroke in diabetics.

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Abbreviations

EPC	Endothelial Progenitor Cells
VEGF	Vascular Endothelial Growth Factor
BDNF	Blood Derived Neurotrophic Factor
LESTR	Leucocyte Derived Seven Transmembrane Receptor
CXCR4	Cysteine-X-Cysteine Receptor 4
SDF-1a	Stromal Cell Derived Factor-1 alpha
MMP-9	Matrix Metalloproteinase-9
VLA	Very Late Antigen
TTC	Triphenyltetrazolium Chloride
MCAO	Middle Cerebral Artery Occlusion
GFAP	Glial Fibrillary Acidic Protein
HIF-1a	Hypoxia Inducible Factor-1 alpha
SVZ	Subventricular Zone
VCAM-1	Vascular Cell Adhesion Molecule-1

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INTRODUCTION AND PURPOSE:

Type II diabetes mellitus affects a huge number of patients worldwide (Wang *et al.*, 2006a). It is a metabolic disorder characterized by insulin resistance and the increase in blood glucose level. It is associated with serious cardiovascular and cerebrovascular complications (Kumari *et al.*, 2007). A serious cerebrovascular complication which is commonly associated with diabetes, is the ischemic stroke, which is considered as one of the leading cause of death and disability all over the globe. The outcomes of ischemic stroke in diabetics are poor with greater mortality and morbidity seen in such patients (Bonow and Gheorghiade, 2004). Successful neovascularization, which is a key step in the repair of any ischemic tissue, has been reported to be impaired in diabetes (Tamarat *et al.*, 2004). This may be a possible reason for the greater severity of ischemic stroke seen in such patients. The angiogenic deficiency in diabetics is attributable to many factors like reduction in number of endothelial progenitor cells (EPCs) (Fadini *et al.*, 2005) and their dysfunctionality (Fadini *et al.*, 2006b) together with the reduced expression of some chemokines responsible for homing and engraftment of the EPCs to the ischemic site (Gallagher *et al.*, 2007). But the exact pathophysiology of this deficiency, causing impairment of tissue repair in diabetics after an ischemic stroke, still remains largely unknown.

Ischemic Stroke and Diabetes:

Ischemic stroke is a neurological deficit of vascular origin lasting 24 hours or longer or until death. In this condition, the supply of blood to the brain is diminished due to a blockade in the cerebral vasculature. Clinical trials like MRFIT and ARIC have shown the increased risk of ischemic stroke in diabetics (Idris *et al.*, 2006). The Baltimore Washington Cooperative Young Stroke Study (1996) has also shown the risk of ischemic stroke to be increased by ten fold in young people (< 44 years) with diabetes compared to normal subjects (Rohr *et al.*, 1996). Outcomes after ischemic stroke are also worse in diabetics as evidenced by higher morbidity and mortality rates and more severe residual neurological deficits, in such patients compared to the normal subjects (Haheim *et al.*, 1995;Oppenheimer *et al.*, 1985;Toni *et al.*, 1992). Conditions associated with diabetes, like obesity, insulin resistance, impaired glucose tolerance (Idris *et al.*, 2006), and also presence of silent cerebral infarcts (Baliga and Weinberger, 2006) collectively constitute important risk factors for stroke. However, the pathophysiology of the worse outcome after ischemic stroke in diabetics is still not well understood.

After an ischemic stroke, the neurons generally die by two processes. They are necrosis and apoptosis(Wang *et al.*, 2001). Necrosis occurs in the core region and is characterized by permanent inflammation of cytoplasm and organelles leading to the destruction of membrane integrity and cell lysis. The area surrounding the core region of necrotic cell death is termed as *penumbra* which undergoes destruction by apoptosis (Astrup *et al.*, 1981;Li *et al.*, 2004). The *penumbra* can be termed as an area of incomplete infarction, which with a constrained blood supply, remains metabolically viable for about 48 hours

after onset of stroke and thereafter undergoes progressive derangement (Heiss *et al.*, 1992). Thus, *penumbra* is a region encompassing the ischemic core which is not only dying after an ischemic event, but also is viable to recover by regeneration of the lost neurons, a process known as neurogenesis (Paciaroni *et al.*, 2009).

The process of neurogenesis can be causally linked to cerebral neovascularization after ischemic stroke (Ohab and Carmichael, 2008;Wang *et al.*, 2006b). The newly formed blood vessels supply nutrition and oxygen to the infarct area, and also secrete factors like angiopoietin-1 which can stimulate neuroblast migration into the peri infarct area (Zhang and Chopp, 2009). Also after stroke, the migration of neuroblasts, expressing phenotype of the mature neurons, to the peri infarct area have been shown to be associated with cerebral vessels (Jiang *et al.*, 2005;Ohab *et al.*, 2006). Besides guiding the migration of neuroblasts, the activated endothelial cells of the blood vessels also secrete vascular endothelial growth factor (VEGF) which helps in neurogenesis (Zhang and Chopp, 2009). The importance of blood vessels in the repair of ischemic brain is further indicated by the correlation of cerebral vascular density and survival time in patients after stroke (Krupinski *et al.*, 1993;Slevin *et al.*, 2000).

Studies indicate that the association between diabetes mellitus and stroke is related to the pathology of cerebral blood vessels, which is prominent in diabetics (Abbott *et al.*, 1987;ALEX *et al.*, 1962;Davis *et al.*, 1987) because cerebral vasculature, as shown earlier, plays an important part in neurogenesis. Diabetes is associated with capillary rarefaction and reduced collateral formation in response to ischemia (Waltenberger, 2001). These can be a probable cause for the increased infarct size seen in type two

diabetic mice after stroke compared to non-diabetic animals (Vannucci *et al.*, 2001). Neovascularization of the adult brain after cerebral ischemia has been shown in experimental stroke and human studies (Krupinski *et al.*, 1993;Zhang *et al.*, 2000). However diabetes reportedly is associated with increased apoptosis of endothelial cells and reduced neovascularization in response to ischemia (Ergul *et al.*, 2009;Fadini *et al.*, 2005;Tamarat *et al.*, 2004).

Neovascularization of adult ischemic tissue can take place in two different ways. They are (i) angiogenesis and (ii) vasculogenesis. In angiogenesis, there is sprouting of mature endothelial cells from the pre-existing vascular network which give rise to new blood vessels in response to hypoxia. In vasculogenesis, bone marrow-derived endothelial progenitor cells in circulation accumulate in the ischemic tissue in response to hypoxic stimulus and take part in new vessel formation (Ceradini and Gurtner, 2005;Tamarat *et al.*, 2004). The bone marrow-derived endothelial progenitor cells (EPCs) are circulating immature cells with the ability to differentiate into mature endothelium, which actively take part in neovascularization (Asahara *et al.*, 1999). During osteogenesis, a periosteal bud composed of capillaries, osteogenic cells and mesenchymal cells penetrate into the cartilage prototype of the future bone. These mesenchymal cells give rise to the fixed tissue elements known as stromal cells (Dorshkind, 1990). These stromal cells, which represent a mixed cell population of various tissue committed stem cells including endothelial progenitor cells (EPCs) are harbored in the hypoxic niche of bone marrow (Li and Chopp, 2009;Suda *et al.*, 2005). Studies suggest that bone marrow derived EPCs make significant contribution to angiogenic growth factor- induced angiogenesis and can also participate in vasculogenesis after cerebral ischemia (Murayama *et al.*, 2002;Zhang

et al., 2002a). Moreover, the EPCs have also been suggested to be involved in neurogenesis of the damaged brain by secretion of neurogenic factors like brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF)(Leventhal *et al.*, 1999;Palmer *et al.*, 2000). Sobrino et al in 2007 also reported the increase in the level of circulating EPCs to be associated with good outcome after acute ischemic stroke, which may be due to the increased neurogenesis induced by the EPCs (Sobrino *et al.*, 2007).

The EPCs, which are thus seen to be important for the repair of the damaged brain after cerebral ischemia, have been reported to be reduced in number and also become dysfunctional in diabetic patients, compared to that in age-matched healthy subjects (Fadini *et al.*, 2005). Glucose tolerance has been shown to be negatively associated with the level of circulating progenitor cells (Fadini *et al.*, 2007a). In vitro study suggests that a high glucose level can induce apoptosis of endothelial cells (Baumgartner-Parzer *et al.*, 1995). Owing to their endothelial phenotype, EPCs may undergo apoptosis when exposed to high glucose, and this may explain in part their decrease in diabetic patients (Fadini *et al.*, 2005). Moreover diabetic EPCs have also been reported to display functional impairments, such as reduced proliferation, adhesion, migration and incorporation into tubular structures (Fadini *et al.*, 2007b;Gallagher *et al.*, 2007;Tepper *et al.*, 2002).

The mechanisms underlying reduction in number and functional impairment of diabetic EPCs, though not clear, may include reduced mobilization and shortened survival of these cells into peripheral blood in diabetes (Fadini *et al.*, 2006a;Fadini *et al.*, 2006b). While the latter has been shown to be dependent on intracellular pathways, triggered by

hyperglycemic stress (Krankel *et al.*, 2005;Seeger *et al.*, 2005), the proper explanation for the former still remains largely unexplored. A recent report shows that mobilization of the EPCs, in response to ischemia is defective in diabetes (Capla *et al.*, 2007). The same report also showed that local injection of normal endothelial cells to the diabetic ischemic tissue failed to restore new blood vessel formation. Reduced mobilization together with the functional impairment of EPCs, as mentioned, may be due to the weakened release of bone marrow stimulating factors like SDF-1a and VEGF in diabetes (Gallagher *et al.*, 2007;Waltenberger, 2001).

These studies suggest that the reduced number of EPCs together with the reduction in factors essential for their proper functioning contributes largely to the impairment of ischemic neoangiogeneis in diabetics, which may be responsible for the worse outcome of these patients after ischemic stroke.

The SDF-1a/CXCR4 axis:

The tissue committed stem cells of the bone marrow including the EPCs have been reported to express Cysteine-X-Cysteine Receptor 4 (CXCR4) on their surface constitutively (Kucia *et al.*, 2005). This CXCR4 is a seven membrane spanning G-protein coupled receptor, originally called LESTR/fusin (Bleul *et al.*, 1996), and got its present designation due to its exclusive binding affinity towards the CXC chemokine, stromal cell derived factor-1 (Li and Ransohoff, 2008). The CXC chemokines represent a subfamily of small chemo attractant proteins that contain four conserved cysteine residues at their amino terminus, of which the first two are separated by a non conserved amino acid (X) (Mehrad *et al.*, 2007). The CXC chemokine, stromal cell derived factor-1

(SDF-1) or CXCL12 was originally isolated from the murine bone marrow in 1993 (Tashiro *et al.*, 1993). The gene for this protein is located in chromosome 10 and is highly conserved across species, maintaining about 99% homology between mouse and man which indicates its fundamental biological significance (Ceradini and Gurtner, 2005). SDF-1 contains two isoforms - alpha (a) and beta (b) - formed by alternative splicing (Stumm *et al.*, 2002) of which the alpha or a is the dominant one. This chemokine is constitutively expressed in high level in the ischemic niche of bone marrow, lymph node and muscle derived fibroblasts (Kucia *et al.*, 2004) and also in tissues like brain and heart in response to ischemia (Chen *et al.*, 2008;Hill *et al.*, 2004)

SDF-1a binds exclusively to its cognate receptor CXCR4 and like any other chemokine, gives rise to downstream pathways which lead to the directional movement of different kinds of cells to the target organ or tissue (Li and Ransohoff, 2008), a process defined as chemotaxis. When SDF-1a binds to the extracellular part of CXCR4, it causes a modification of the tertiary structure of the receptor protein, which in turn triggers the activation of the intracellular part of the heterotrimeric G protein (Rot and von Andrian, 2004). Exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by the activated heterotrimeric G protein leads to its dissociation into α and βγ sub units (al-Aoukaty *et al.*, 1996;Kuang *et al.*, 1996). The α subunit causes activation of phospholipase C which generates diacyl glycerol (DAG) and inositol 1,4,5 triphosphate (IP3) triggering intracellular release of Ca++ ions from endoplasmic reticulum (Li and Ransohoff, 2008). The βγ subunit on the other hand activates phosphoinositide-3 kinase (PI3K) which in turn triggers downstream pathways mediated by serine/threonine kinase AKT, PyK2 and nuclear transcriptional factor (NFκB) (Rot and von Andrian, 2004). The

SDF-1a/CXCR4 interaction has also been suggested to activate signaling pathways like P38 MAPKs and JAK/STAT (Vila-Coro *et al.*, 1999).

The SDF-1a/CXCR4 interaction is transient and shuts down rapidly. The G alpha subunits show intrinsic GTPase activity and hydrolysis of GTP reunites the subunits, again, forming the original inactive heterotrimers (Rot and von Andrian, 2004). Also the binding of SDF-1a to its receptor CXCR4, stimulates phosphorylation of the intracellular part of the CXCR4 receptor by G receptor kinase (GRK) which facilitates its binding to β arrestin (Aragay *et al.*, 1998;Barlic *et al.*, 1999;Fan *et al.*, 2001). This causes internalization of the receptor in the cell (Huttenrauch *et al.*, 2002). Within the cell the receptor is either degraded or resensitized again and transported to the cell surface by a group of enzymes called Rab GTPases (Li and Ransohoff, 2008;Rot and von Andrian, 2004).

Study by Hyduk et al. in 2007 has shown, that increased phospholipase C and intracellular calcium levels, induced by SDF-1a/CXCR4 interaction, greatly enhanced the arrest of transplanted human endothelial progenitor cells to the ischemic myocardium of mice (Hyduk *et al.*, 2007). Also Zhao et al in 2008 has shown the dependence of SDF-1a mediated chemotaxis of human breast cancer cells on activation of PI3K/AKT pathways (Zhao *et al.*, 2008). Massa et al in 2006 has shown that the increase of intracellular calcium and activation of PyK 2 signalling pathway by SDF-1a in $CXCR4^+$ pituitary cells led to the proliferation of these cells in vitro (Massa *et al.*, 2006). The JAK/STAT pathway triggered by SDF-1a/CXCR4 interaction (Vila-Coro *et al.*, 1999) has also been shown to be important for migratory and angiogenic responses of $CXCR4^+$ progenitor

cells (Valdembri *et al.*, 2002;Zhang *et al.*, 2001). So these studies indicate that the interaction of SDF-1a with its cognate receptor CXCR4, may give rise to important intracellular pathways which can play a highly significant role in the proliferation and chemotaxis of $CXCR4^+$ progenitor cells to the region of ischemia, thus facilitating repair.

SDF-1a/CXCR4 axis mediated ischemic tissue repair :

Restoration of vascular supply to the region of ischemic injury is important for their repair (Goligorsky *et al.*, 2009). Endothelial progenitor cells (EPCs) have been suggested to induce reendothelialization in the ischemic area and thus facilitate repair (Asahara *et al.*, 1999;Hristov *et al.*, 2003). But migration and subsequent engraftment of these circulating stem cells in the ischemic tissues are indispensible for the functional recovery after an ischemic injury (Orlic *et al.*, 2001). SDF-1a, a chemokine expressed in a wide variety of tissues under low oxygen tension (Kucia *et al.*, 2004;Shirozu *et al.*, 1995), is a strong chemoattractant for the $CXCR4^+$ haematopoeitic stem cells and helps their successful migration from fetal liver to the bone marrow (Zou *et al.*, 1998). The EPCs are thought to be originated from hematopoietic stem cells of the bone marrow (Goligorsky *et al.*, 2009) and also express CXCR4 receptor on their surface (Kucia *et al.*, 2005). This together with the fact that SDF-1a is upregulated in tissues after an ischemic attack (Chen *et al.*, 2008;Hill *et al.*, 2004) indicates that SDF-1a/CXCR4 pathway may be involved in attraction of the $CXCR4^+$ EPCs to the ischemic tissue and thus facilitate repair.

Under normal physiological conditions, SDF-1a is highly expressed in the hypoxic niche of bone marrow than in any other tissue (Chang *et al.*, 2007). Expression of SDF-1a in hypoxic environment has been attributed to the expression of another transcription factor

called hypoxia inducible factor-1alpha (HIF-1a) because the binding domain of the latter has been shown to be present on the promoter region of SDF-1a gene (Ceradini and Gurtner, 2005). The expressed SDF-1a remains attached to the endothelial cells of the bone marrow via heparin sulphate proteoglycan on the luminal surface of these cells (Middleton *et al.*, 2002). The CXCR4 receptor, present on the EPCs of the bone marrow (Kucia *et al.*, 2005), interacts with the SDF-1a, and this interaction leads to the activation of the integrin molecule, very late antigen-4 (VLA-4) on the surface of EPCs, resulting in the firm adhesion of these cells to the hypoxic niche of bone marrow (Lataillade *et al.*, 2004).

Following injury SDF-1a is upregulated in the ischemic tissue (Hill *et al.*, 2004) and is first released in the intravascular compartment. It is then translocated in the plasma and ultimately reaches the bone marrow compartment through a process called transcytosis (Dar *et al.*, 2005). After entering the bone marrow microenvironment the SDF-1a activates several proteases like elastase, cathepsin G and matrix metalloproteinase-9 (MMP-9) (Lataillade *et al.*, 2004;Petit *et al.*, 2007). The proteases disrupt the interaction between SDF-1a and $CXCR4^+$ EPCs in the bone marrow by N-terminus cleavage of SDF-1 (McQuibban *et al.*, 2001). This is followed by reduction in the expression of VLA-4 on the surface of EPCs (Mohle *et al.*, 1993). These, alongwith the increased levels of SDF-1a in the peripheral blood following ischemic injury (De *et al.*, 2004), mobilize the EPCs to the systemic circulation (Petit *et al.*, 2007),(Heissig *et al.*, 2002).

After entering the systemic circulation the endothelial progenitor cells should get directed towards the ischemic site. The migration of $CXCR4^+$ cells towards regions

expressing SDF-1a has been suggested to be dose dependent (Fischer *et al.*, 2004). The endothelial cells at the site of ischemia highly express SDF-1a (Yao *et al.*, 2003). This high gradient of SDF-1a in the ischemic site thus guides the migration of endothelial progenitor cells (EPCs) towards the ischemic areas (Yamaguchi *et al.*, 2003).Once they reach the site of ischemia the $CXCR4^+$ EPCs interact with SDF-1a expressed by the ischemic endothelium. This interaction leads to the activation of integrins like VLA-4 and VLA-5 on the surface of EPCs (De *et al.*, 2004) and also vascular cell adhesion molecule (VCAM-1) on the damaged endothelium (Butler *et al.*, 2005). The expression of these integrins and adhesion molecule leads to the adhesion of EPCs to the vascular wall (Massberg *et al.*, 2006). The activation of integrins by SDF-1a/CXCR4 interaction also stimulates the reorganization of actin which in turn helps the arrested cells to undergo transendothelial migration (Nishita *et al.*, 2002;Peled *et al.*, 2000). After infiltration, the $CXCR4^+$ EPCs take up their position in the perivascular niche where there is a high concentration of SDF-1a (Petit *et al.*, 2007). This high concentration of SDF-1a also helps in the retention of these angiogenic cells in the ischemic niche (Jin *et al.*, 2006), and stabilization of the newly formed blood vessels (Petit *et al.*, 2007).

The SDF-1a/CXCR4 axis can hence be an important pathway for the neovascularization of the ischemic tissue and the reduced expression of any of its components can lead to impairment of tissue repair after an ischemic attack (Schober *et al.*, 2003;Schober *et al.*, 2006;Zernecke *et al.*, 2005).

Ischemic tissue repair in diabetes: SDF-1a/CXCR4 axis dysfunction :-

It has been observed in animal models that diabetes decreases vascular density is reduced after hind limb ischemia (Abaci *et al.*, 1999;Yarom *et al.*, 1992). Neovascularization, which is important for the repair of ischemic tissue gets severely impaired in diabetes (Fadini *et al.*, 2006b;Tamarat *et al.*, 2004). Reduced collateral formation in response to ischemia, as is evident from the angiograms of diabetic patients, can lead to circulatory deficits which may impair the repair of ischemic tissues in them (Abaci *et al.*, 1999). Poor collateral formation in diabetes may be attributed to the reduced mobilization of stem cells from the bone marrow (Fadini *et al.*, 2007a;Tamarat *et al.*, 2004).

The $CXCR4^{+}$ endothelial progenitor cells (EPCs), harbored in the hypoxic niche of bone marrow, which are important for the neovascularization of the ischemic tissue, are reduced in number and display functional impairments like decreased proliferation, adhesion, migration and incorporation into vascular structures in diabetes (Fadini *et al.*, 2007a;Fadini *et al.*, 2006b;Gallagher *et al.*, 2007;Tepper *et al.*, 2002). The defective homing and recruitment of diabetic EPCs have also been shown by Capla *et al.* in an in vivo murine model. The same group also found that injection of normal endothelial progenitors to the streptozotocin induced diabetic mice could not restore the formation of new blood vessels to the baseline (Capla *et al.*, 2007). These results indeed suggest a weakened release of certain angiogenic factors like SDF-1a and vascular endothelial growth factor (VEGF), can be a probable cause for the functional impairment of EPCs seen in diabetes (Ceradini and Gurtner, 2005;Gallagher *et al.*, 2007).

The EPCs express CXCR4, the cognate receptor for SDF-1a. But the expression of SDF-1a has been reported to be diminished in the peripheral wounds of diabetic mice (Gallagher *et al.*, 2007). Hypoxia inducible factor-1a (HIF-1a) expressed under ischemia has been suggested as the upstream signal for the transcription of SDF-1a, since the promoter region of SDF-1a gene shows binding domain for HIF-1a (Ceradini and Gurtner, 2005;Schober and Zernecke, 2007). The expression of HIF-1a is in turn up regulated by the cytokine called insulin like growth factor-1 (Yu *et al.*, 2007), which has been reported to be diminished in the diabetic wounds (Bitar, 2000;Brown *et al.*, 1997). This can thus decrease HIF-1a in diabetic wounds which can in turn be a probable reason for the decreased secretion of SDF-1a in diabetes (Ceradini and Gurtner, 2005). Also the activity of the enzyme, dipeptidyl peptidase IV has been shown to be enhanced in diabetes compared to those obtained from normal subjects (Segal *et al.*, 2006). This is an enzyme that cleaves off and inactivates SDF-1a (Ohtsuki *et al.*, 1998;Perrault *et al.*, 2004). The increased activity of this enzyme in diabetes indicates another possible reason for the functional impairment of SDF-1a chemokine in diabetes. Beside this, the level of $CXCR4^{+}$ progenitor cells have also been reported to be diminished in the peripheral blood of patients with diabetic complications (Egan *et al.*, 2008).

So these studies collectively suggest that there may be multiple defects in the intrinsic expression of SDF-1a/CXCR4 axis in diabetes which may be an important factor for the diabetic impairment of wound healing after ischemic injury.

Regenerative therapy of stroke in diabetes : Significance of SDF-1a/CXCR4 axis :-

Diabetes is associated with poor outcome after ischemic stroke as is evident from the greater mortality and morbidity seen in type two diabetic patients after stroke (Ho *et al.*, 2003;Kurukulasuriya *et al.*, 2006;Spratt *et al.*, 2003) . Studies with diabetic patients and rodent models have shown increased cellular death and impaired recovery of the ischemic tissue after stroke (Folbergrova *et al.*, 1992;Siesjo, 1988). Restoration of the lost blood vessels to the ischemic area is important for the recovery after an ischemic stroke because the newly formed blood vessels can supply oxygen and nutrients required for the repair (Zhang and Chopp, 2009) . The newly formed blood vessels also supply neurogenic factors, guide the migration of neuroblasts from the sub ventricular zone of brain to the ischemic area and helps in their proliferation and maturation (Zhang *et al.*, 2000;Zhang *et al.*, 2002b;Zhang and Chopp, 2009). Thus the process of neovascularization in the ischemic brain can be causally linked to neurogenesis (Ohab *et al.*, 2006;Wang *et al.*, 2006b) which is associated with better functional outcome after stroke (Bliss *et al.*, 2007;Zhang and Chopp, 2009).

Evidence that intravenous administration of bone marrow derived stromal cells, can induce angiogenesis in the ischemic boundary after stroke (Chen *et al.*, 2001a), has fostered active investigation for using these cells in the cell transplantation therapy of ischemic stroke. Intrastriatal transplantation of non hematopoietic stem cells from the bone marrow, has been reported to improve functional recovery after stroke in a murine model (Li *et al.*, 2000). Transplantation of EPCs, a member of the family of bone marrow derived stem cells, has improved blood flow and vascular density in ischemic hind limb

of mice (Kalka *et al.*, 2000). In addition to neovascularization, EPCs have also been shown to improve nerve conduction (Naruse *et al.*, 2005) and the level of circulating EPCs has been associated with better outcome after ischemic stroke (Sobrino *et al.*, 2007).

Expression of the CXCR4 receptor on the surface of EPCs (Kucia *et al.*, 2005;Mohle *et al.*, 1998) and upregulation of its specific ligand, SDF-1a, in the brain after focal cerebral ischemia (Hill *et al.*, 2004;Stumm *et al.*, 2002), makes SDF-1a/CXCR4 an attracting therapeutic target for ischemic stroke. SDF-1a has been shown to be involved in mobilizing of $CXCR4^+$ progenitor cells (Sweeney *et al.*, 2002) from the bone marrow and the subsequent homing of these cells to the site of ischemic injury (Ceradini and Gurtner, 2005;Sweeney *et al.*, 2002). Also, local delivery of SDF-1a gene in the ischemic hindlimb of mice has been shown to promote angiogenesis (Hiasa *et al.*, 2004;Yamaguchi *et al.*, 2003) because apart from being a strong chemoattractant for $CXCR4^+$ EPCs (Aiuti *et al.*, 1997;Hattori *et al.*, 2001;Peled *et al.*, 1999), the SDF-1a, itself has been shown to induce VEGF expression which promotes angiogenesis in vivo (Salcedo *et al.*, 1999). Also SDF-1a has been reported to be involved in the attraction of $CXCR4^+$ neuroblasts from the sub ventricular zone (SVZ) in the ischemic boundary of adult rodent brains after cerebral ischemia (Zhang and Chopp, 2009) which indicates its role in neurogenesis.

The physiological expression of SDF-1a in response to ischemia is however transient (Hill *et al.*, 2004) and often insufficient for ischemic repair in disorders like diabetes (Gallagher *et al.*, 2007). Transplantation of bone marrow derived stromal cells, has been shown to increase the concentration of SDF-1a, and cause subsequent migration of

neuroblasts to the peri infarct area after stroke in animal models (Chen *et al.*, 2003a;Shen *et al.*, 2007). Specially engineered human bone marrow derived stem cells, when transplanted intravenously, have been shown to overexpress neurotrophic factors which have reduced cerebral ischemic damage in rat model, following focal cerebral ischemia (Kurozumi *et al.*, 2005). However, functional recovery with the cell transplantation therapy after stroke, is associated with the number of transplanted cells homing to the ischemic part, and even after intracerebral transplantation very few cells have been found to integrate in the ischemic brain (Bliss *et al.*, 2007). Thus increasing the factors, responsible for the homing of the transplanted cells to the ischemic site can largely contribute to the success of cell transplantation therapy after ischemic stroke.

SDF-1a, a main homing factor for endogenous as well as transplanted progenitor cells, has been reported to be diminished in diabetic wounds (Gallagher *et al.*, 2007) alongwith the reduction in $CXCR4^{+}$ progenitor cells (Egan *et al.*, 2008). Thus investigating the expression of the SDF-1a/CXCR4 pathway in the diabetic brain after ischemic stroke can be useful in making the cell transplantation therapy in such patients more successful.

Hypothesis: We hypothesize that SDF-1a/CXCR4 axis is impaired in type II diabetic (db/db) mice, and this dysfunction is responsible for enlarged damage and poor outcome after ischemic stroke in diabetic mice.

Specific Aim-1: To determine the expression of SDF-1a/CXCR4 and cerebrovascular density in db/db diabetic mice and its control db/+ mice.

Specific Aim-2: To determine the infarct volume and the expression of SDF-1a/CXCR4 in db/db diabetic mice and its control db/+ mice in response to ischemic stroke induced by middle cerebral artery occlusion surgery (MCAO).

Specific Aim-3: To determine the over expression of SDF-1a and its localization in the brains of db/db mice, injected with AAV-SDF-1a vector.

Experimental Design.

Specific Aim 1: Male db/db diabetic mice of age 8 weeks and age matched non-diabetic db/+ mice (n = 4/group) were used for the experiment. Mouse were transcardially perfused with PBS and 4% paraformaldehyde under anaesthesia and brains were collected. Microvascular density in the frontal cortex were determined by immunohistochemical analysis in both groups and the results were quantitatified by Image-J software (NIH).

Specific Aim 2: Male db/db diabetic mice of age 8 weeks and age matched non-diabetic db/+ mice (n = 12/group) were used for the experiments. Focal cerebral ischemia was induced in both groups by middle cerebral artery occlusion surgery (MCAO). 48 hours after surgery the mice from each group were divided into two subgroups (n = 6/group). One subgroup containing both db/db and db/+ mice were perfused under anesthesia and brains were collected and sectioned using cryostat (1 mm thickness). Images of the sections were taken by confocal microscope (Leica TCS SP2) and volume of infarction was quantified with Image J software (NIH). The other sub group containing both db/db and db/+ mice (n = 6/group) were sacrificed and both the ischemic and non ischemic sides of the brains were divided into two halves. One half was collected in RNA later and the other half was collected in dry ice. Western blot was done for determining the

expression of proteins, SDF-1a and CXCR4 with half of brains collected in dry ice for both the ischemic and non ischemic sides. Real time RT PCR was done with half of brains collected in RNA later for determining the expression of m-RNA for SDF-1a and CXCR4.

Specific Aim 3: Male db/db diabetic mice of age 8 weeks (n = 6) were used for the experiment. Mice were anesthetized and received microinjection of Adeno associated virus, AAV-null and AAV-SDF-1a, into left and right side of the striatum respectively. Brains were harvested for 14 days after injection and the expression of SDF-1a was determined by immunohistochemical analysis. The localization of SDF-1a in brain was determined by double immunohistochemical analysis with antibodies for SDF-1a and glial fibrillary acidic protein (GFAP). Images were obtained by confocal microscopy (Leica TCS SP2).

Materials and Methods:

Animals. C57BL/6 strain of mice have been widely used as animal models for ischemic stroke (Tamaki *et al.*, 2006). C57BLKS/J strain of mice has 70% homology to the C57BL/6 strain. Adult male C57BLKS/J Cgm+/+$^{Leprdb/J}$ mice (Jackson Labs, USA) of age 8 weeks served as our murine model for type-2 diabetes (db/db) and their lean littermates (db/+) served as controls. Mice were fed with standard chow (Harlan) and water ad libitum. All procedures were approved by the Wright State University Laboratory Animal Care and Use Committee (LACUC) and were in accordance with the *Guide for the Care and Use of Laboratory Animals* issued by the National Institute of Health.

Methods:

Determination of microvascular density:

Microvascular density in the frontal cortex of brain was measured in both db/db and db/con group as per previous reports(Munzenmaier and Greene, 2006;Oyamada *et al.*, 2008). The db/db (diabetic) and db/+ (control) were injected with Ketamine/xylazine mixture (100:8 mg/kg). Mice were perfused transcardially with phosphate buffer saline (PBS) to flush away the blood and then with 4% paraformaldehyde to fix the microvessels in brain. The brains were then kept in 4% paraformaldehyde overnight at 4 degree centigrade. The brains were cryo protected with 30% sucrose in PBS for three days. After 3 days sections of the frontal cortex (20 μm) from both the groups were cut

using cryostat and washed with PBS for 3 times 5 mins each. Free floating sections (20μm) were mounted on slides and they were blocked with 5% normal donkey serum (Vector Lab) in PBS containing 0.3% Triton-X 100 and 1% BSA (bovine serum albumin, Sigma) for 1 hour at room temperature. Sections were then incubated overnight with primary anti body for CD31 cells, rat anti CD31 (1:50, BD Biosciences) diluted in PBS with 0.3% Triton X-100 and 1% BSA. The sections were washed with PBS for 3 times 10 mins each and were then incubated for 2 hrs. at room temperature with Alexa Fluor 594-conjugated donkey anti rat IgG (1:200, Invitrogen) secondary antibody. After 2 hours the slides were washed in PBS for 30 mins and the sections were coverslipped with fluorescence mounting medium (Vector Lab, H-1000). Following this the slides were visualized with a confocal microscope (Leica TCS SP2). Five adjacent images (1 mm^2) were taken from each of the three sections in the frontal cortex region and quantified with image J software (NIH).

Middle Cerebral Artery Occlusion Surgery:

Middle Cerebral Artery Occlusion (MCAO) is a common method of inducing focal ischemic stroke to mice and has been described previously (Hata *et al.*, 1998;Walther *et al.*, 2002). Mice were anesthetized with isoflurane (1-5 %) inhalation using precision vaporizer. Incision was made along the neck. One drop of buprinorphine (0.25% buprinorphine) was applied locally for analgesia. Left common carotid artery was exposed and legated. The external carotid artery was legated and cut off to expose the left interior carotid artery. A 7-0 nylon monofilament (Ethicon, Somerville, NJ, USA) with

heat- blunted tip (220 μm), coated with silica was inserted through an incision on common carotid artery and advanced into left interior carotid artery to reach the base of the left middle cerebral artery (about 10 mm distal to the carotid bifurcation). The suture was left in position to induce permanent MCA occlusion (Fig-1). The skin incision was rejoined with an uninterrupted suture using 5.0 Dexon II sterile suture with CE-2 cutting needle. Immediately after surgery mice were treated with buprinorphine (0.1mg/kg s.c.). The same dose of buprinorphine was administered the first and second day after surgery for analgesia.

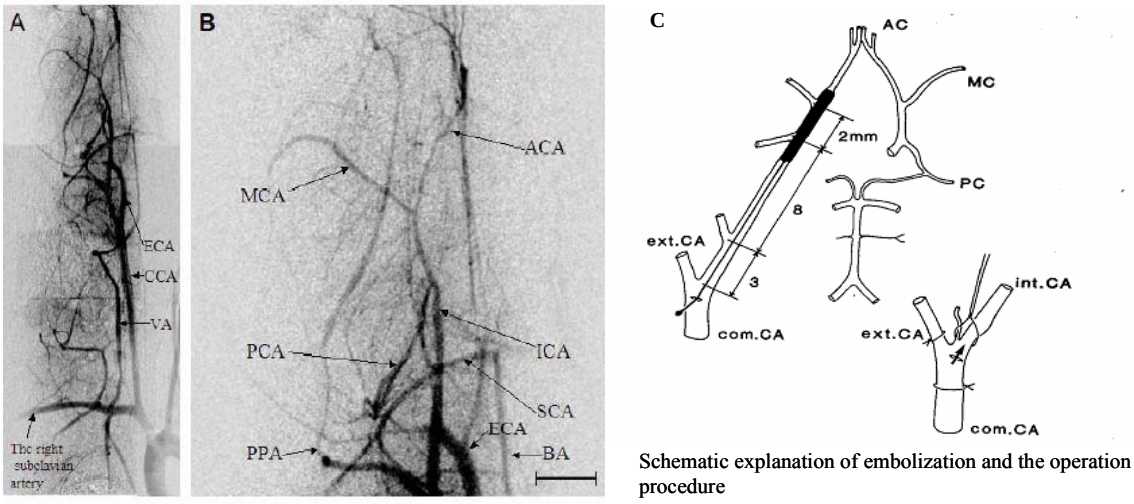

Figure 1. An assembled view of (A) brain and neck and an original image with skull in normal C57BL/6 mice. (B) ACA; anterior cerebral artery, BA; basilar artery, CCA; common carotid artery, ECA; external carotid artery, ICA; internal carotid artery, MCA; middle cerebral artery, PCA; posterior cerebral artery, PPA; pterygopalatune artery,

SCA; superior cerebellar artery, VA; vertebral artery. (C) The MCAO surgery represented schematically by passing a nylon monofilament through an incision on the common carotid artery (Tamaki *et al.*, 2006).

Determination of cerebral infarct volume:

Cerebral infarct volume was determined by staining with 2,3,5 triphenyl tetrazolium chloride. TTC staining is an established method for quantifying cerebral infarction (Bederson *et al.*, 1986). TTC is a salt which accepts a proton from succinate dehydrogenase in the inner membrane of mitochondria and subsequently gets reduced to a red insoluble form formazan. Thus the infarct area that is an area of inactive enzymes does not get stained and appears pale (Tureyen *et al.*, 2004). Mice that underwent MCAO surgery were sacrificed 48 hours after the surgery. Brains were removed and cut into 5-6 sections with a mouse brain matrix. Each section was of thickness 1 mm. The brain sections were stained with 2% solution of TTC (Lundy *et al.*, 1986) for 25 minutes at 37 degree centigrade in the LC incubator in dark. Sections were checked every 5 mins to confirm the stain. The sections were then washed with saline and fixed in 4% paraformaldehyde for 30 mins at room temperature. They were stored at 4 degree centigrade in 10% formalin before analysis. Images of stained slices were taken using a flat bed scanner and were saved in the computer for analysis. The border between infarct (unstained) and non-infarct (stained) tissue was outlined with the image-J software (NIH), an image analysis system. The infarct area was calculated by subtracting the area of non-lesioned ischemic hemisphere from the total area of the slice. The volume of

infarction was calculated by integration of lesion areas (area of infarct [mm^2] x thickness [1mm]) (Lin *et al.*, 1993).

Western Blot Analysis of SDF-1a and CXCR4 expression in brain :

This technique was performed for the determination of SDF-1a and CXCR4 protein expression in the ischemic and non ischemic sides of brains of both db/db and db/+ mice after MCAO surgery. The mice were sacrificed by decapitation and the brains were collected in dry ice 48 hours after surgery. The samples from all the groups were thawed and homogenized in 400µl of cold lysis buffer which contained glycerol, HEPES (pH 7.4), EDTA, NaCl, MgCl2 and protease inhibitor (Roche Diagnostics, Indianapolis,USA). The samples were sonicated and centrifuged for 5 minutes at 14,000g. The supernatants were recovered from each tube and the protein concentrations were determined by the Bradford method for protein estimation using Bio Rad reagent (Bio Rad Laboratories, Hercules, CA). For gel loading, the final sample volume was corrected to 20µg of total protein. To each of the samples, 10µl of loading buffer (40% glycerol, 50% Tris-HCL, 10% SDS, bromophenol blue, 0.4% β-mercaptoethanol) was added and the samples were boiled for 5 minutes. Then the samples were loaded on 10% Tris-Glycine SDS-Page gel (BioRad Laboratories, Hercules, CA). Gels were electrophoresed at 100V, 50 mA until the dye front came near the bottom of the gel. The proteins were transferred from the gel to 0.2µm PVDF membranes overnight at 10V and 50mA. The PVDF membranes were blocked with 10 ml of 5% milk in TBS-T buffer for 1 hour at room temperature. Then primary antibodies were added. Rabbit Anti-CXCR4 (Ana Spec, San Jose, CA, USA) 1:500 was used as the primary antibody for CXCR4. For

SDF-1a, monoclonal mouse anti SDF-1a antibody 1:200 (R&D Systems Inc.,USA) was used. β-actin was used as a house keeper. For that, monoclonal anti β-actin(Sigma, Saint Louis,MO, USA) was used with a dilution of 1:4000 with both SDF-1a and CXCR4. Primary antibodies were diluted in blocking buffer for a total of 10ml/membrane. The membranes were probed with primary antibody for 2 days at 4^{o}C. Then the membranes were washed with TBST solution for 3 times, 5 mins. each. The membranes were probed with secondary antibody for 1 hour at room temperature. The secondary antibodies were diluted to 1:40000 (1µl/40 ml) with TBS-T buffer. For SDF-1a, horse radish peroxidase linked antibody, anti mouse CXCL12 were used. For CXCR4, anti rabbit CXCR4 were used. After probing, the membranes were washed again with TBST and the sample proteins were viewed via the chemiluminescence produced by the ECL substrate (Pierce) and captured by the Fuji LAS3000 imager. The relative amounts of protein of interest (POI) were determined by normalizing to β-actin.

Normalized Value : $(POI_{density}- Background_{density})/(Actin_{density}-Background_{density})$

No values were excluded from the final data calculations unless one of the bands was missing. This method of simultaneous probing is a well established one and has been described previously (Mouihate *et al.*, 2002).

Real Time Reverse Transcriptase PCR analysis of SDF-1a and CXCR4 expression in brain:

This procedure was performed for confirming the expression of m-RNA for SDF-1a and CXCR4 in brains of both the db/db and db/con mice as per previous reports (Cook *et al.*, 2004;Gallagher *et al.*, 2007). The mice were sacrificed by decapitation and the brains

were collected in RNA later. The procedure was divided into three parts: (i) Extraction of RNA from the respective tissues (ii) Reverse transcription of the RNA to form cDNA (iii) Amplification and quantification of cDNA by Real time PCR with SDF-1a and CXCR4 primers.

(i) Extraction of RNA:

The RNeasy mini kit was obtained from Qiagen Sciences, Maryland, USA. The kit contained Buffer RLT, Buffer RPE, 70% ethanol, Buffer RDD and Buffer RW_1. The buffers were first prepared for use according to the manufacturer's protocol. For this 1 ml of buffer RLT was mixed with 10µl of β-mercaptoethanol. 44 ml of 100% ethanol was added to buffer RFE. 70µl of buffer RDD was added to 10µl DNAse. The tissues were first homogenized using buffer RLT under a rotor stator.600µl of buffer RLT was added to the tubes for brain. The tissues were homogenized. Then they were microcentrifuged at max. speed for 5 mins. at room temperature and the supernatant were transferred to a new set of tubes.600µl of 70% ethanol was added to the tubes containing the homogenized brains. The tubes were gently inverted to mix immediately. Then 700µl of each sample, including the pellets if formed, were transferred to the RNeasy mini spin column sitting on a 2 ml collection tube. The tubes were centrifuged for 25 secs at 8000 x g.The flow through was discarded. 700µl of buffer RW_1 (wash buffer) was pipetted into the RNeasy column. The tubes were closed gently and centrifuged for 15 minutes at 8000g or 10,000 rpm at room temperature. The flow through was discarded alongwith the collection tube. Then DNase-1 stock solution was made. For that 550µl of RNAse free water was added to the lyophilized DNase vial. From this 10µl was added to 70µl of buffer RDD and was mixed by gentle inversion. The lyophilized DNase incubation mix (80µl) was pipetted

directly into the RNeasy silica-gel membrane and placed for 30min-1hour at room temperature. 350µl of buffer RW_1 was pipetted into the RNeasy mini column and was placed for 1 min. It was centrifuged at 8000g or 10000 rpm for 15 secs and the flow through was discarded. 500µl of Buffer RPE (wash buffer) was pipetted into the RNeasy column and the tube was closed gently. It was centrifuged at 8000g or 10000 rpm for 15 secs and the flow through was discarded. 500µl of buffer RPE was again pipetted to the RNeasy column. The tubes were closed gently and centrifuged at 8000g or 10000 rpm for 2 mins. to dry the RNeasy silica gel membrane. The RNeasy column was transferred to a new set of 1.5 ml collection tube.35µl of RNase free water were added to the tubes. They were kept for 5 mins. and centrifuged at 8000 x g for 2 mins to elute. Nano Drop ND-1000 spectrophotometer was used to measure the concentration of the RNA in the eluted samples.

(ii) Reverse Transcription of RNA to form cDNA :

Complementary DNA or cDNA is a double stranded DNA that is derived from mRNA obtained from prokaryotes and eukaryotes. It is convenient to act with the cDNA because it is not easily degraded by omnipresent RNases. So m-RNA obtained from the tissues were reverse transcripted with high capacity cDNA archive kit (Qiagen Life Sciences, Maryland, USA). For this technique, the reagents used were 10x Buffer RT, dNTP mix, primers for SDF-1a and CXCR4 (50µM), RNase inhibitor (20 unit/µl), omniscript reverse transcriptase, template RNA. The master mix was prepared with these reagents as per manufacturer's protocol. Template RNA of brain from both the control and test

samples were then added to the respective tubes. The tubes were incubated for 1 hour at 37 degree centigrade in PCR machine.

(iii) Amplification of cDNA with SDF-1a and CXCR4 primers and quantification by real time reverse transcriptase PCR:

Three groups of tubes, two of which contained cDNA for both control and test samples, were diluted 20 fold with RNase-free water. Master mixture was made with Fast SYBR Green Master Mix (2X) and both reverse and forward primers for SDF-1a and CXCR4 respectively in separate groups of tubes as per manufacturer's protocol. The primer sequences used were: for CXCR4 (5'-CTC CAA GGG CCA CCA GAA-3' and 5'-GGC AAA GAA AGC TAG GAT GAG G-3') (Invitrogen, USA), for SDF-1a (5'-CCA GAG CCA ACG TCA AGC AT-3', 5'-TGT TGA GGA TTT TCA GAT GCT TGA-3') (Invitrogen, USA), and for GAPDH (F- 5'-TGC ACC ACC AAC TGC TTA G-3' and R-5'-GAG GGG CCA TCC ACA GTC TTC TG-3') (Invitrogen, USA). The cDNA from control and test samples were transferred to the respective tubes. The third group of tubes contained the PCR master mix with primers for the housekeeper, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) against which the levels of both SDF-1a and CXCR4 were to be determined and standardized. After mixing well by a brief vortex the tubes were centrifuged in Beckman Coulter 6R centrifuge for 1 min. at 1000 rpm at a temperature of 20 degree centigrade. Then the tubes were subjected to real-time RT PCR reaction in a sequence detection system (Applied Biosystems 7500) for 1 hour 23 minutes. The amplification plot was obtained. The threshold cycle values (Ct) for SDF-1a, CXCR4 and GAPDH were calculated using a sequence detection software, 7500

systems SDS software (Applied Biosystems). The relative expression of m-RNA was obtained using the $2^{-\Delta\Delta CT}$ method according to a previous report (Livak and Schmittgen, 2001) .

Determination of overexpression of SDF-1a in brain after microinjection of AAV-SDF-1a into brain :

To develop the method for overexpression of SDF-1a in brain, microinjection of adeno associated virus containing SDF-1a gene was performed according to a previous report (Cearley and Wolfe, 2007). The AAV-SDF-1a and AAV-Null vectors were obtained from Dr. Yiaolian Tang (Keck Graduate Institute, USA) (Tang *et al.*, 2005). The vectors were packaged into AAV9 virus serotype by University of Penn Vector Center (titer, 1.6x 10^{13} GC/ml). The mice were placed on the stereotaxic frame, with the head placed flat, for microinjection of AAV-SDF1a or AAV-null into left striatum. An incision was made on the back head (around 1 cm). A plastic cannula (Plastics One) was used for guidance of injection. A small hole (1 mm diameter), pointing the injection location was drilled into the skull for the insertion of a glass micropipette (50 μm). The guide was lowered close to the hole. Then, the glass micropipette was lowered through the guide cannula and the hole into the striatum (-0.5 mm caudal to bregma, 2.0 mm lateral, and 2.5 mm ventral). The outside part of the glass micropipettes were connected to a picospritzer system using a suitable catheter. All injections were 1 μl over 30 seconds and left in place for 1 minute to minimize upward flow of viral solution after raising the micropipettes.

For determining the overexpression of SDF-1a immunohistochemical analysis was performed. 14 days after injection of the vector, the mice were injected with Ketamine/xylazine mixture (100:8 mg/kg). Mice were perfused transcardially with PBS

to flush away the blood and then with 4% paraformaldehyde. The brains were fixed in 4% paraformaldehyde overnight at 4 degree centigrade. The brains were cryo protected with 30% sucrose in PBS for three days. After 3 days brains were sectioned using cryostat and sections were collected in PBS. Free floating medial sections (20μm) were blocked with 5% normal donkey serum (Vector Lab) in PBS containing 0.3% Triton-X 100 for 1 hour at room temperature. Sections were then incubated overnight with primary antibody for SDF-1a, the monoclonal anti human/mouse SDF-1a (R&D Systems Inc.USA) diluted to 1:50 concentration by the blocking buffer. The sections were washed with PBS for 3 times 10 mins each and were then incubated for 2 hrs. at room temperature with Alexa Fluor - conjugated donkey anti rat IgG (1:200, Invitrogen) secondary antibody. After 2 hours the sections were washed in PBS for 30 mins, mounted on slides and were coverslipped with fluorescence mounting medium (Vector Lab, H-1000). Following this, the slides were visualized with a confocal microscope (Leica TCS SP2).

Determination of localization of SDF-1a in the glial cells of brain by double immunohistochemistry after microinjection :

Double immunohistochemistry with antibodies for SDF-1a and glial fibrillary acidic protein (GFAP), a protein associated with the glial cells, was performed for determining the transfection of SDF-1a in the astrocytes of brain of mice injected with AAV-SDF-1a according to a previous report with slight modification. Mice were injected with ketamine/xylazine mixture (100:8 mg/kg) and were perfused transcardially with PBS to

flush away the blood and then with 4% paraformaldehyde. The brains were then fixed in 4% paraformaldehyde, overnight at 4 degree centigrade. The brains were cryoprotected with 30% sucrose in PBS for three days. After 3 days brains were sectioned using cryostat and sections were collected in PBS. Free floating medial sections (20 μm) were blocked with 5% normal donkey serum (Vector Lab) in PBS containing 0.3% Triton-X 100, for 1 hour at room temperature. Sections were then incubated overnight with primary antibodies for SDF-1a and GFAP, respectively, monoclonal anti human/mouse SDF-1a (R&D Systems Inc) and chicken anti GFAP antibody (1:250, Chemicon, USA). Following this, the sections were washed with PBS and incubated in secondary antibodies, Alexa Fluor 488 donkey anti mouse IgG (1:100, Invitrogen) for SDF-1a and Alexa Fluor 594 donkey anti chicken (1:100, Invitrogen) for two hours at room temperature. After two hours the sections were washed in PBS for 30 mins, mounted on slides and were coverslipped with fluorescence mounting medium (Vector Lab, H-100). Following this, the slides were visualized with a confocal microscope (Leica TCS SP2). The expression of SDF-1a and GFAP were visible at wave lengths of 488 nm and 594 nm respectively.

Statistical analysis: All statistics have been represented as mean ± SE. The data of microvascular density and the volume of ischemic damage were analyzed by using paired student's t test. Data of western blot and real time RT PCR were assessed by one way ANOVA followed by Fisher LSD post hoc test. Differences between groups have been considered significant when $p<0.05$ for all procedures.

Results :

1) Body weight and blood glucose level in both db/+ and db/db mice:

Body weight and blood glucose level were determined in both diabetic and the control group of mice 24 hours before surgery. The level of plasma glucose was determined using an Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostic, IN, USA) Table-1 shows the significant increase of both body weight and glucose level in the diabetic group compared to the controls.

TABLE-1:

Variants	db/+	db/db
B.W. (g)	23.5 ± 0.3	37.5 ± 0.9 *
Blood glucose (mg/dl)	179.7 ± 16.5	524.1 ± 10.2 *

*: P< 0.0001, compared with db/+; B.W.: Body weight ; n = 6/group

2) Determination of micro vascular density in the brains of db/+ and db/db mice by immunohistochemical analysis:

The micro vascular density in the frontal cortex region of both diabetic (db/db) and control (db/+) mice were determined using confocal microscopy and quantified with Image J software (NIH). The density (no. of capillaries/mm^2) was found to be reduced in the cortex of diabetic mice as compared to the controls (n=4/group, paired t test, Fig 2)

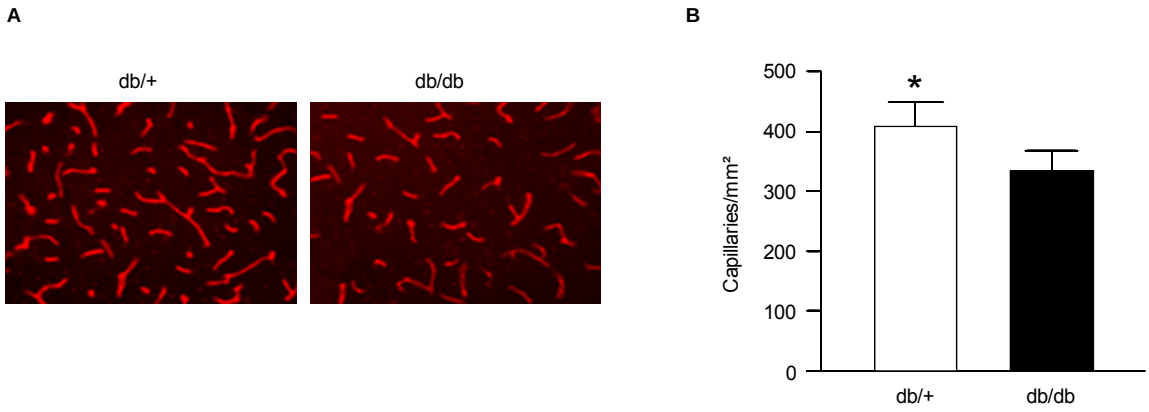

Figure-2. (A) Images of the frontal cortex of diabetic (db/db) and control (db/+) mice. Note the reduced vascular density in the diabetic group. (B) The graph shows the significant reduction in the number of capillaries per square millimeter in the diabetic group. (*p<0.05, n=4/group)

3) TTC staining for determination of volume of ischemic damage in brains of db/db and db/+ mice :

One millimeter thick coronal sections of brains from both db/+ and db/db mice were stained with 2,3,5- triphenyltetrazolium chloride (TTC), 48 hours after permanent occlusion of middle cerebral artery. Non ischemic areas of both groups have converted TTC to a deep formazan compound and thus appear dark red in figure while ischemic areas have failed to metabolize TTC and appear white in figure (A) which shows the enlarged ischemic damage in the db/db mice compared to the db/+ mice after MCAO surgery. Morphometry of ischemic area has been determined with TTC staining and expressed as the percentage of total area in db/+ and db/db groups (B). (n=4/group, paired t test, Fig 3)

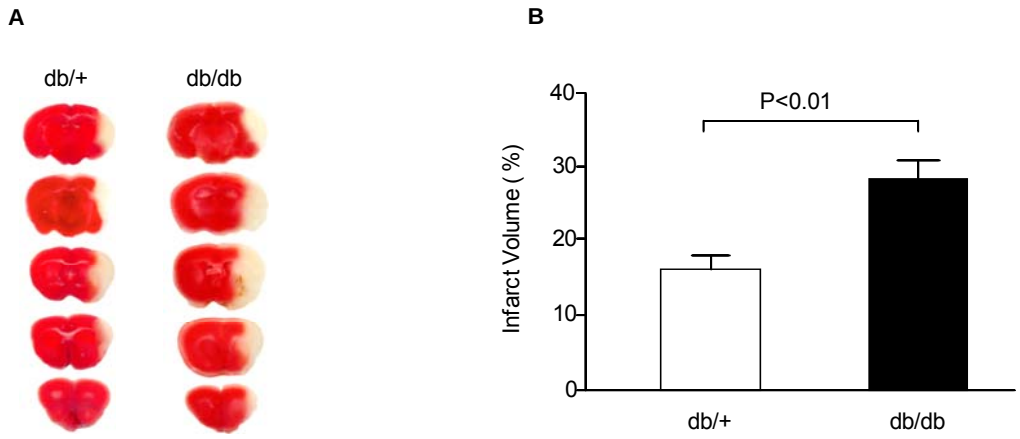

Figure-3. (A) TTC stained coronal sections of brain from control (db/+) and (db/db) mice subjected to permanent middle cerebral artery occlusion for 48 hrs. (B) Morphometry of ischemic area determined with TTC staining and expressed as a percentage of total area in both db/db and db/+ mice. Values are mean ± SE. ($p<0.01$, n=4/group)

4) Expression of SDF-1a protein in the brains of db/+ and db/db mice after focal cerebral ischemia:

The expression of the protein SDF-1a was increased in the ischemic side compared to the non ischemic side of both the db/+ and db/db mice but the increase was significantly more in db/+ mice compared to that of db/db mice in both the sides (n = 6/group, one way ANOVA with Fisher LSD post hoc test; Fig. 4)

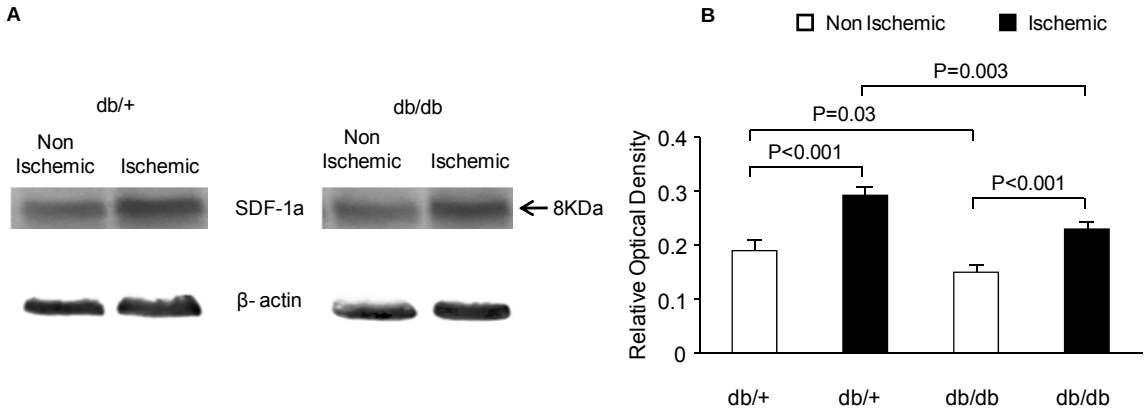

Figure-4. Comparison of SDF-1a expression in the non ischemic and ischemic sides of brains of both db/db and db/+ mice, 48 hours after middle cerebral artery occlusion surgery. (A) Gel showing the increased expression of SDF-1a in the ischemic sides as compared to non ischemic sides of both db/+ and db/db mice. (B) Densiometric analysis showing significantly increased SDF-1a expression in both sides of db/+ mice compared to db/db mice. ($p < 0.05$, n = 6/group)

5) Expression of CXCR4 protein in the brains of db/+ and db/db mice after focal cerebral ischemia:

The expression of CXCR4 was increased in the ischemic side compared to non ischemic sides of both the db/+ and db/db mice but the increase was significantly more in db/+ mice compared to that of db/db mice only in the ischemic hemisphere. In non ischemic side the difference of expression of CXCR4 between the two groups, was not significant. (n = 6/group, one way ANOVA with Fisher LSD post hoc test; Fig. 5)

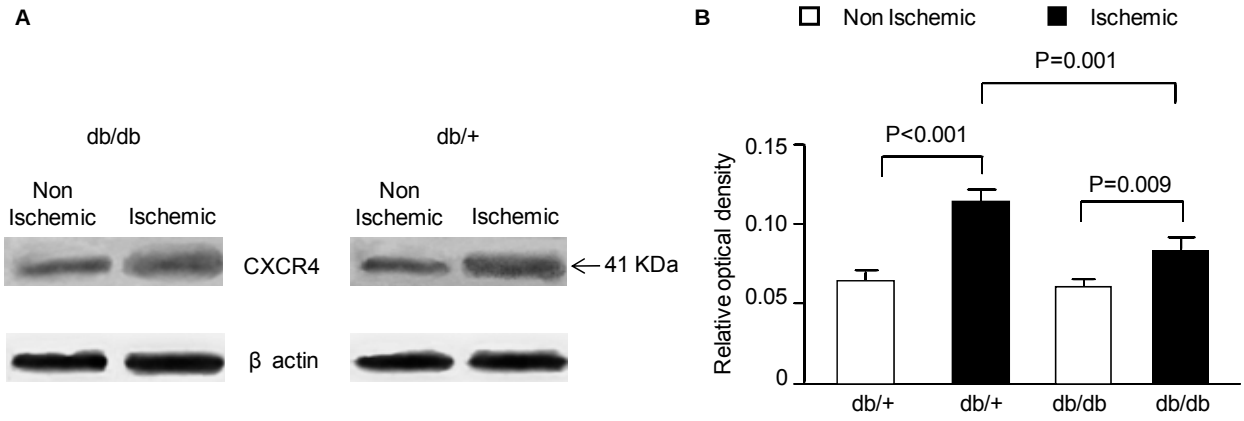

Figure-5. Comparison of CXCR4 expression in the ischemic and non ischemic sides of brains of both db/+ and db/db mice, 48 hours after middle cerebral artery occlusion surgery. (A) Gel showing the increased expression of CXCR4 in the ischemic side compared to non ischemic sides of brains of both db/+ and db/db mice. (B) Densiometric analysis showing significantly increased CXCR4 expression in db/+ mice compared to db/db mice only in the ischemic side. ($p<0.05$, n=6/group)

6) Expression of mRNA for SDF-1a as determined by Real-Time RT-PCR:

The expression of m-RNA for SDF-1a was increased in the ischemic side compared to the non ischemic side of both the db/+ and db/db mice but the increase was significantly

more in db/+ mice compared to that of db/db mice in both the sides (n = 6/group, one way ANOVA with Fisher LSD post hoc test; Fig. 6)

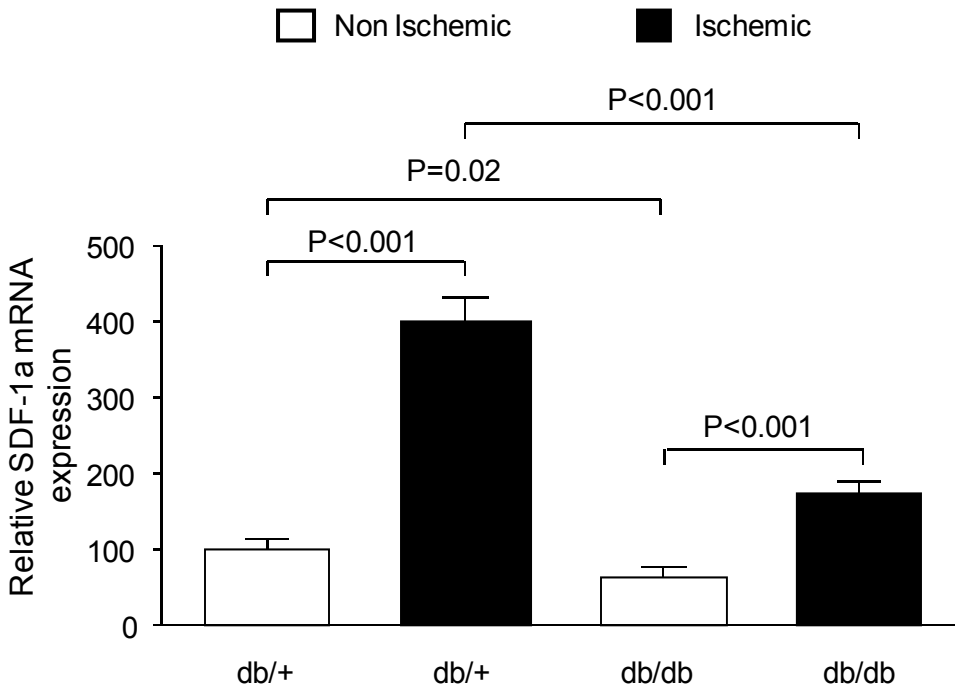

Figure-6. Comparison of m-RNA expression for SDF-1a in the non ischemic and ischemic sides of brains of both db/db and db/+ mice. Graph showing significant increase in SDF-1a m-RNA expression in both sides of db/+ mice compared to db/db mice. ($p < 0.05$, n = 6/group)

7) Expression of mRNA for CXCR4 as determined by Real-Time RT-PCR:

The expression of m-RNA for CXCR4 was increased in the ischemic side compared to non ischemic sides of both the db/+ and db/db mice but the increase was significantly more in db/+ mice compared to that of db/db mice only in the ischemic side. In non ischemic side the difference of expression of CXCR4 between the two groups, was not significant (n = 6/group, one way ANOVA with Fisher LSD post hoc test; Fig.7)

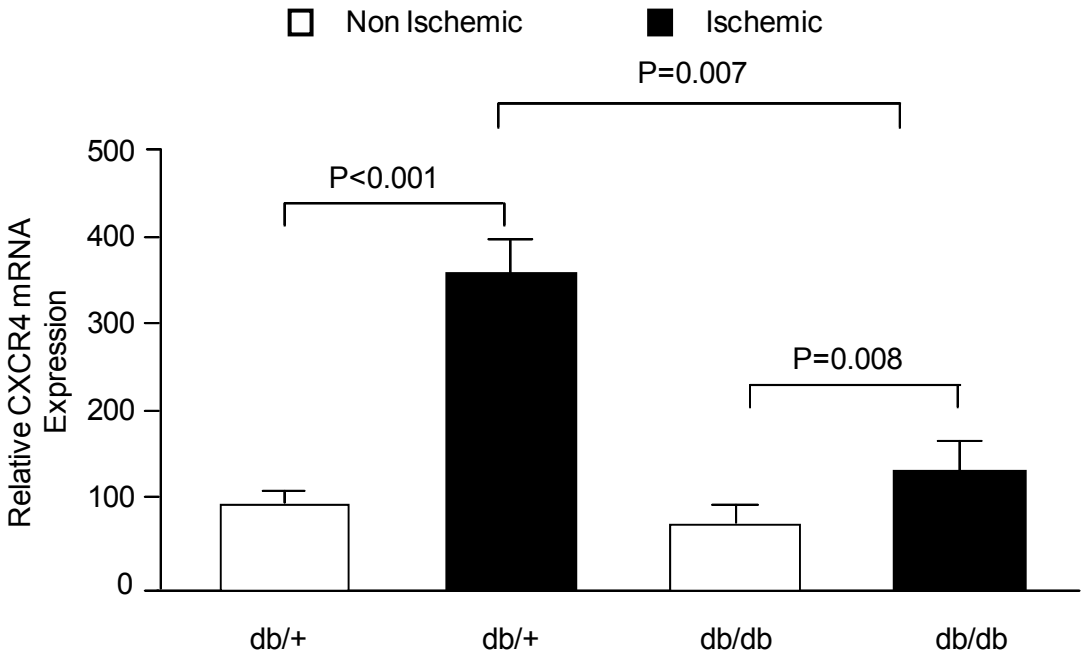

Figure-7. Comparison of expression of m-RNA for CXCR4 in the ischemic and non ischemic sides of brains of both db/+ and db/db mice. Graph showing significantly

increased CXCR4 m-RNA expression in db/+ mice compared to db/db mice only in the ischemic side. ($p < 0.05$, n = 6/group)

8) Overexpression of SDF-1a in the brain striatum of db/db mice :

The db/db diabetic mice (n = 6) received microinjection of AAV-SDF-1a in the right striatum and AAV-null in the left striatum. Immunohistochemical analysis shows the successful overexpression of SDF-1a in the part receiving AAV-SDF-1a. (Fig-8).

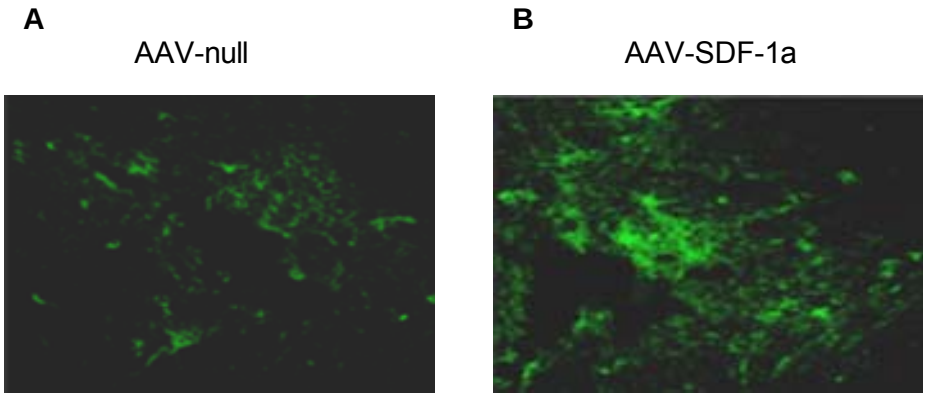

Figure- 8. Overexpression of SDF-1a in the striatum of mouse brain. (A) Image of left striatum of mouse brain showing the expression of SDF-1a after injection of AAV-null. (B) Image of right striatum of mouse brain showing the over expression of SDF-1a after injection of AAV-SDF-1a.

9) Overexpression of SDF-1a in glial cells after microinjection of AAV-SDF-1a :

The db/db diabetic mice (n = 6) received microinjection of AAV-SDF-1a in the right striatum and AAV-null in the left striatum. Double immunohistochemistry was done with antibodies for SDF-1a and glial fibrillary acidic protein (GFAP). Confocal microscopy shows overexpression of SDF-1a and GFAP in the right striatum (Fig- 9II) compared to the left striatum (Fig- 9I). The merged image (arrow-marked) also shows higher level of SDF-1a localizing in the glial cells in the right striatum (Fig- 9II) compared to the left striatum (Fig- 9I)

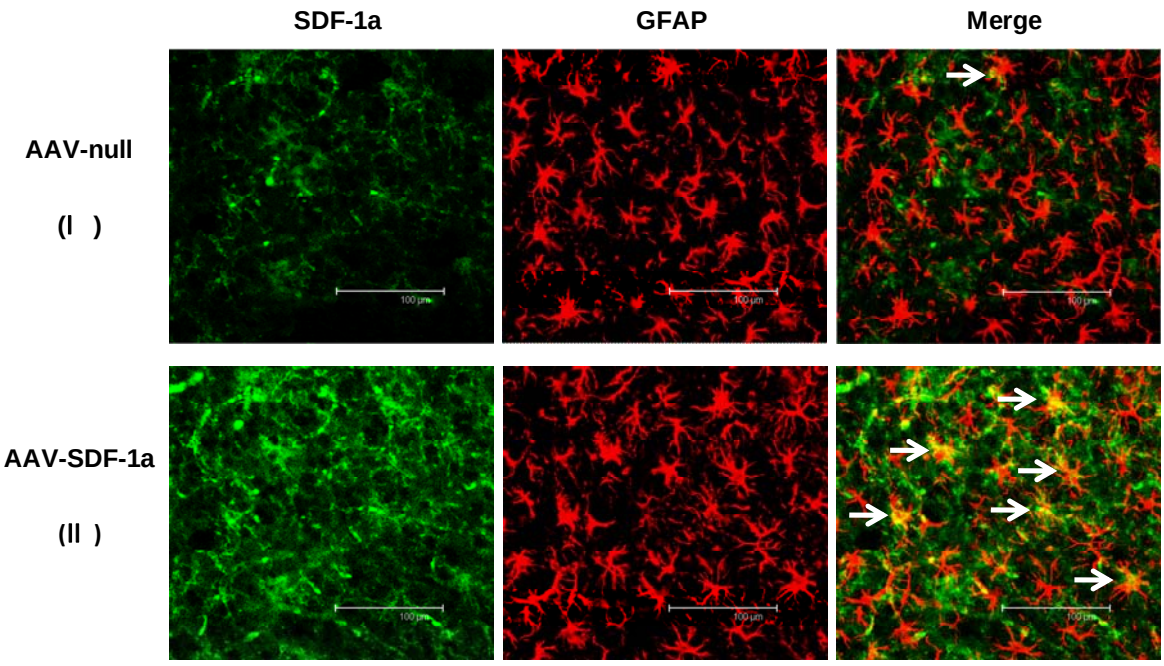

Figure- 9. Confocal images of right striatum and left striatum of the brain of db/db mice receiving microinjection of AAV-SDF-1a and AAV-null respectively. Note the overexpression of SDF-1a and GFAP in the right striatum (Fig- 9 II) compared to the left striatum (Figs- 9I). The merged image also shows higher level of SDF-1a loacalizing in the glial cells in the right striatum (Fig- 9 II) compared to the left striatum (Fig- 9I). Scale bar 100 μm.

DISCUSSION

Cardiovascular complications like congestive heart failure and stroke account for about 80% of excess deaths in patients suffering from type two diabetes mellitus (Kurukulasuriya *et al.*, 2006). Clinical trials have shown the increased incidence and poor outcome of ischemic stroke in diabetics (Idris *et al.*, 2006). Angiographs of diabetic patients have shown impaired neovascularization in the heart, after myocardial ischemia (Abaci *et al.*, 1999). The endothelial progenitor cells (EPCs) which take part in neovascularization of the ischemic tissue have been reported to be dysfunctional and reduced in number in diabetes (Fadini *et al.*, 2005). The factors responsible for the dysfunction of EPCs in diabetes, are however not clear. The EPCs express CXCR4 receptors on their surface and thus show preferential migration to tissues expressing their specific ligand SDF-1a (Li and Chopp, 2009). This together with the earlier reports of the upregulation of SDF-1a in the ischemic tissue after injury (Gallagher *et al.*, 2007;Hill *et al.*, 2004), suggests the SDF-1a/CXCR4 axis to be a possible pathway for promoting mobilization and homing of stem cells to the injured tissue after ischemia. However, most of the previous studies have focused on the involvement of this axis in the process of peripheral wound healing (Ceradini and Gurtner, 2005;Gallagher *et al.*, 2007).

In this study, we have determined the microvascular density and the volume of infarction after ischemic stroke in db/db diabetic mice. We have also studied the expression of SDF-1a/CXCR4 axis in the ischemic and non ischemic hemispheres of brain of 8 weeks old diabetic (db/db) mice both at the levels of protein and mRNA.

We first determined the cerebral microvascular density in the frontal cortex of db/db diabetic and the age-matched normal db/+ mice. The endothelial cells of the microvessels express CD31 markers on their surface and so we used rat anti CD31 to stain the microvessels in the brain. We found that the microvascular density was significantly decreased in the brains of db/db mice compared to that in the db/+ mice (Fig- 2). Our data showed the reduced microvascular density in the diabetic mice indicating a general reduction in tissue vascularization in diabetes. Vascularization is important for tissue blood supply from dominated and collateral vasculatures, and also an important step in the process of repair after an ischemic damage because the newly formed blood vessels supply oxygen and nutrients which are necessary for the regeneration of the lost tissue. Diabetes has been shown to be associated with decreased collateral vessel formation in response to ischemia (Weihrauch *et al.*, 2004), suggesting impaired neoangiogenesis in diabetics after ischemic injury. This impaired angiogenesis can causally link to impaired neurogenesis after stroke in diabetics because angiogenic blood vessels secrete factors like angiopietin-1 which can stimulate neuroblast migration in the infarct area after stroke (Ohab and Carmichael, 2008). Thus impairment of angiogenesis can eventually lead to a poor outcome after ischemic stroke in diabetes.

Secondly, we determined the severity of ischemic damage in db/db diabetic mice after inducing permanent focal cerebral ischemia via middle cerebral artery occlusion surgery. We used TTC for staining the brain sections. Our results (Fig- 3) showed the significant enlargement in the volume of infarction in the brains of diabetic mice as compared to the control mice, indicating a greater severity of ischemic damage in the diabetic group. This poor outcome after ischemic stroke in diabetic mice has been supported earlier also by

the work of Cakmak et al 2007 who showed the increased infarct volume in streptozotocin induced diabetic mice after middle cerebral artery occlusion surgery (Cakmak *et al.*, 2007) and Kusaka et al in 2004, whose group reported the infarct size to be double in the STZ induced diabetic mice compared to the controls (Kusaka *et al.*, 2004)

In this study we used the focal cerebral ischemic model because it closely resembles the human stroke and produces pathological lesions which are heterogeneous and have both necrotic core and salvageable penumbra and also normal tissue in both the ipsilateral and contralateral hemispheres (Kuraoka *et al.*, 2009). Different methods are available for inducing permanent focal cerebral ischemia in mice. The most common methods are middle cerebral artery occlusion (MCAO) via intraluminal suture or direct ligation (Xi *et al.*, 2004). In our experiment we used the intraluminal suture method to avoid the hassles of craniotomy associated with direct ligation. 2,3,5- triphenyl tetrazolium chloride (TTC) staining was used as a quantitative method for the comparison of the severity of ischemic stroke in diabetic mice and the controls. TTC staining is an established method for the detection and quantification of cerebral ischemia in experimental animals (Bederson *et al.*, 1986) TTC is a water soluble salt which itself is not a dye but gets reduced by the mitochondrial enzymes of normal tissue into a fat soluble red light sensitive compound called formazan which turns the normal tissue red, while keeping the infarct tissue unstained because of the unavailability of the active mitochondrial enzymes. It is a reliable marker to differentiate the ischemic and non ischemic part of a tissue and has been shown to be effective up to 3 days after ischemic stroke (Bederson *et al.*, 1986).

Earlier reports suggest that revascularization of the injured part following an ischemic attack is an important step in the process of recovery. Circulating endothelial progenitor cells, derived from the bone marrow, have been shown to mediate endothelial regeneration and neovascularization after tissue ischemia (Sobrino *et al.*, 2007). Studies suggest that EPCs take part in the cerebral revascularization in adult brain after ischemia (Zhang *et al.*, 2002a). Increase of circulatory EPCs in the first week of injury have been associated with good functional outcome and reduced infarct size in patients after acute ischemic stroke (Sobrino *et al.*, 2007). The expression of the CXCR4 receptor on the surface of the EPCs have been shown to be important for the mobilization of these progenitors into the blood and their successful homing to the site of injury after myocardial ischemia (Zhang *et al.*, 2008). In diabetes, the circulating EPCs show considerable dysfunctionality and reduction in number (Fadini *et al.*, 2005). Fadini et al in 2006 has shown the ischemia induced mobilization of EPCs to be defective in streptozotocin induced diabetic mice (Fadini *et al.*, 2006b). As because the expression of CXCR4 receptor is important for the mobilization and homing of EPCs to the ischemic site and is correlated with the number of EPCs accumulating to the site of ischemic injury, we went ahead to determine the expression of CXCR4 protein in the ischemic and non ischemic parts of the brains of diabetic mice and their controls. Our results of western blot (Fig- 5) showed a significant decrease in the CXCR4 protein expression on the ischemic part of the brains of diabetic mice as compared to the controls. But in the non ischemic part of the brains, though the diabetic mice showed a slight decrease in CXCR4 protein expression compared to the controls, the difference was not significant. To verify the results we got by western blot, we went on to determine the expression of

CXCR4 at the level of m-RNA by real time reverse transcriptase PCR. Results (Fig- 7) showed that m-RNA for CXCR4 also follows a similar pattern of expression as that of the protein. That is, the expression of m-RNA for CXCR4 was found to be significantly lowered in the ischemic part of the brains in diabetic mice compared to the controls but in the non ischemic part the decrease of CXCR4 m-RNA was not significant. This reassures the reduced expression of CXCR4 in the brains of type 2 diabetic mice in response to focal cerebral ischemia which also indicates a decreased homing of $CXCR4^+$ EPCs to the ischemic brain in diabetes. Our results are in line with the works of Egan et al. who in 2008 reported the reduced expression of CXCR4 in peripheral blood mononuclear cells of diabetic patients (Egan *et al.*, 2008).

For successful repairing of the ischemic tissue after stroke the circulating $CXCR4^+$ EPCs should home to the ischemic region. The work of Capla et al. in 2007 suggested a reduced recruitment of the EPCs to the ischemic site in type two diabetic patients when compared to normal subjects (Capla *et al.*, 2007). The CXCR4 receptor expressed on EPCs show high affinity for binding to their exclusive ligand SDF-1a (Li and Ransohoff, 2008). Kollet et al. in 2003 have shown increased homing and engraftment of human bone marrow derived progenitor cells in murine liver by the injection of SDF-1a (Kollet *et al.*, 2003). The work of Hill et al in 2004 have suggested the upregulation of the chemokine SDF-1a in the ischemic penumbra following stroke and its importance in the homing of $CXCR4^+$ cells of the bone marrow to the areas of ischemic injury (Hill *et al.*, 2004). SDF-1a has also been reported to be associated with the migration of neuroblast into the periinfarct area of brain (Chen *et al.*, 2003b). SDF-1a, which is thus an important

factor for the homing of $CXCR4^+$ cells to the site of injury has been shown to be reduced in the peripheral wounds of diabetic mice (Yamaguchi *et al.*, 2003).

The aforesaid reports provide a good rationale for investigating whether the expression of the chemokine SDF-1a gets reduced in the brains of diabetic mice in response to ischemic stroke. So we went ahead to measure SDF-1a, both at the levels of protein and m-RNA, expressed in the brains of diabetic mice and the controls, forty eight hours after the induction of ischemic stroke by MCAO. Our results of western blot (Fig- 4) and real time RT PCR (Fig- 6) clearly show the decreased expression of the chemokine, SDF-1a, in the brains of the diabetic mice as compared to their controls, both at the protein and m-RNA levels.

The reduced expression of SDF-1a and its cognate receptor CXCR4 may thus indicate an intrinsic defect of the SDF-1a/CXCR4 axis in type 2 diabetes which may be responsible for the worse outcome of ischemic stroke associated with the patients suffering from this disease. In this study we have focused on the expression of SDF-1a and its receptor CXCR4 in the brains only. Further studies are required to investigate the expression of this axis in the bone marrow of the diabetic mice which may throw light on the reduced mobilization of the $CXCR4^+$ EPCs from the hypoxic niche of bone marrow to the systemic circulation, seen commonly in diabetics.

Cell transplantation therapies show promise in the treatment of ischemic stroke. The stromal cells from bone marrow are a good choice for these therapies because these cells not only differentiate into neuronal and endothelial cells in the ischemic brain (Yamaguchi *et al.*, 2003) but also secrete angiogenic, neurogenic and antiapoptotic

factors in the hypoxic environment of the infarct area (Sun *et al.*, 2003). Animal models like that of Chen et al in 2003 has shown the induction of angiogenesis and the resulting functional benefits obtained by the intravenous administration of human bone marrow stromal cells in rats (Chen *et al.*, 2001b). The $CXCR4^{+}$ EPCs which come under the family of bone marrow stromal cells have been reported to improve blood flow recovery and vascular density in ischemic hind limb of mice, after transplantation by intracardiac injection (Kalka *et al.*, 2000). This coupled with the fact that SDF-1a is upregulated in the ischemic tissue after stroke (Hill *et al.*, 2004) indicates a positive role of the SDF-1a/CXCR4 axis in the repair of ischemic tissue after injury.

Given the comprehensive reduction of SDF-1a/CXCR4 axis in diabetes as shown by our study and also studies done by other groups, strategies increasing the expression level of SDF-1a in the brain may offer new avenues for treatment of ischemic stroke in diabetes.

Reports by Ceradini et al in 2005 and Peled et al in 2000 showed that a constant concentration gradient of SDF-1a is a prerequisite for the successful homing of EPCs in the hypoxic tissue (Ceradini and Gurtner, 2005;Peled *et al.*, 2000). Physiological upregulation of SDF-1a in response to ischemia is however transient (Hill *et al.*, 2004) and has been reported to be diminished in diabetic wounds (Gallagher *et al.*, 2007). So increasing the level of SDF-1a in the ischemic brain might induce the homing of EPCs and improve the impaired healing of ischemic stroke in diabetics. Badillo et al in 2007 have reported the lentiviral mediated gene transfer of SDF-1a to be beneficial in diabetic wound healing. They successfully accomplished the local transfer of SDF-1a gene into the peripheral wounds of diabetic mice and found complete epithelialization by two

weeks (Badillo *et al.*, 2007). Earlier Tang et al in 2005 has also achieved successful mobilization of haematopoietic stem cells into ischemic myocardium by plasmid mediated transfer of SDF-1a in mice(Tang *et al.*, 2005).

So based on the previous reports, we explored the development of a method for Adeno associated virus (AAV) mediated delivery of SDF-1a gene in the brains of diabetic mice. The vectors, AAV-SDF-1a and AAV-Null, were packaged into AAV9 virus serotype since the AAV9 vector in a recent study has been shown to successfully overexpress carried gene in ipsilateral striatum, cortex and hippocampus (Cearley and Wolfe, 2007).The mice received microinjection of AAV-Null into the left and AAV-SDF-1a in the right striatum of brain. Subsequent immunohistochemistry (Fig- 8) showed significant overexpression of the SDF-1a gene in the right striatum of the mice brain compared to the left striatum suggesting the success of the procedure.

We also determined the localization of the SDF-1a protein in the brain of diabetic mice after microinjection. By double immunohistochemistry with antibodies for both SDF-1a and glial fibrillary acidic protein (protein associated with glial cells), we were able to show clearly the localization of SDF-1a in the glial cells of brain (Fig- 9). Earlier works by Buffo et al in 2008 have shown that glial cells can acquire stem cell activity after brain injury and takes part in neurogenesis (Buffo *et al.*, 2008). Successful over-expression of SDF-1a in the brain after microinjection AAV-SDF-1a and its association with the glial cells, as shown in our study, thus indicates a possible use of AAV-SDF-1a for the repair of the ischemic brain after stroke. The upcoming results from our laboratory will throw

light on the effects of the plasmid mediated delivery of SDF-1a on neurogenesis and angiogenesis of the injured brain after ischemic stroke.

CONCLUSION

These results demonstrate that the SDF-1a/CXCR4 axis is dysfunctional in db/db diabetic mice, which is evidenced by the decreased expression at basal and reduced upregulation in response to ischemia. The impaired SDF-1a/CXCR4 axis may be responsible for the reduced microvascular density in the cortex of db/db mice. These abnormalities together may consequently account for the enlarged ischemic damage in the db/db diabetic mice and targeting the functional improvement of SDF-1a/CXCR4 axis can thus offer a new therapeutic avenue for ischemic stroke in diabetes.

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