Published in final edited form as:

Transl Stroke Res. 2016 August ; 7(4): 248–260. doi:10.1007/s12975-016-0464-8.

Impact of Comorbidities on Acute Injury and Recovery in Preclinical Stroke Research: Focus on Hypertension and Diabetes

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Abstract

Human ischemic stroke is very complex and no single preclinical model can comprise all the variables known to contribute to stroke injury and recovery. Hypertension, diabetes and hyperlipidemia are leading comorbidities in stroke patients. The use of predominantly young adult and healthy animals in experimental stroke research has created a barrier for translation of findings to patients. As such, more and more disease models are being incorporated into the research design. This review highlights the major strengths and weaknesses of the most commonly used animal models of these conditions in preclinical stroke research. The goal is to provide guidance in choosing, reporting and executing appropriate disease models that will be subjected to different models of stroke injury.

INTRODUCTION

It has recently been estimated that 90% of the population attributable risk of ischemic stroke is due to 10 known risk factors and include, hypertension (HTN), current smoking, obesity, poor diet, inactivity, diabetes mellitus, high alcohol intake, psychosocial stress and depression, cardiac causes and lipid abnormalities [1]. Of these risk factors, HTN is the most important contributor to overall risk [1] and has been reported in 77% of patients experiencing their first stroke [2]. Diabetes, although less common, is a potent contributor to ischemic stroke risk, especially in younger individuals. Of note, the incidence of diabetes in stroke patients increased dramatically from 1997 (20%) to 2006 (30%) and the combination of hypertension and diabetes is particularly potent when it comes to elevated stroke risk [2].

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The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

Disclosure/Conflict of Interest

Adviye Ergul declares that she has no conflict of interest.

Sherif Hafez declares that he has no conflict of interest.

Abdelrahman Fouda declares that he has no conflict of interest.

Susan C. Fagan declares that she has no conflict of interest.

Stroke epidemiology data does not routinely identify the numbers of ischemic stroke patients with NO vascular risk factors, but it is definitely less than 23%.

In contrast, in a comprehensive review of preclinical evaluation of 502 experimental therapies for acute focal ischemic stroke, only 10% were tested in animals with hypertension. Hypertensive animals have larger infarct sizes with similar durations of ischemia and usually experience reduced efficacy upon therapeutic intervention, compared to normotensive animals [3]. In addition, hypertensive animals have been shown to demonstrate exaggerated response to agents with hypotensive potential [4]. Even fewer preclinical investigations assess the effects of diabetes or acute hyperglycemia on response to therapeutic intervention. Recent interest in the impact of diabetes on preclinical stroke outcome has revealed that targets for intervention differ in animals with comorbid diabetic vascular disease [5] and these animals may actually respond better to some therapeutic interventions [6].

In order to improve the translatability of preclinical stroke research to the stroke patient, experts have recommended evaluation of interventions in animals with comorbid disease [7]. Beyond that, no recommendations exist on how to achieve this goal. Animal models of hypertension and diabetes are numerous and introduce new sources of variability and cost that are difficult to glean from published reports. For example, both genetically hyperglycemic Goto-Kakizaki (GK) rats and genetically hypertensive spontaneously hypertensive rats (SHR), gain weight more slowly than their healthy counterparts, making it necessary to include comparator animals based on weight or age, but not both. Also, careful modification of the method of occlusion may need to occur in order to assure comparable degrees of blood flow reduction in animals of different mean weights [8]. In the case of chronic hyperglycemia, the duration and magnitude of the elevated blood glucose is an important determinant of lesion size and stroke outcome [9].

In the following review, we will include recommendations regarding the conduct of preclinical animal studies in hypertension and diabetes, based upon the published literature. We aim to assist investigators in selecting animal models and designing experiments to address the impact of comorbid vascular disease on ischemic stroke outcome.

I. Hypertension

A. Animal models of hypertension in stroke research—Given the clinical importance of hypertension in the pathogenesis of stroke, laboratories have incorporated hypertensive animals in their preclinical stroke studies. Analyzing studies conducted in hypertensive animals using middle cerebral artery occlusion (MCAO - the most widely used model of ischemic stroke), more than half of the studies were done using SHRs. This is followed by stroke-prone renovascular hypertensive rats (RHRSP) and stroke-prone spontaneously hypertensive rats (SHRSP), respectively (Fig. 1).

SHRs are widely used as a genetic model of hypertension. They were developed by Okamoto and colleagues in Japan in 1963 through selective inbreeding of hypertensive Wistar-Kyoto (WKY) rats until a uniform colony of hypertensive animals was achieved [10]. Being a genetic model of hypertension, they have been proposed to model essential

hypertension in humans. Yet their similarities to essential hypertension have been a subject of debate [10]. Essential hypertension represents 90% of cases of hypertension [11], but the genes involved in human essential hypertension may not be the same as those involved in SHR. Furthermore, different mechanisms could be involved in the development of hypertension in humans versus SHR [10]. SHR have neural and cerebrovascular changes that are different from normotensive rats and may mimic the changes in the cerebrovasculature of people with genetic hypertension. Yet, these changes could also be attributed to the genetic makeup of these rats and thus not exclusively explained by the elevated blood pressure (BP) [12]. However, SHR are by far the most widely used model of experimental hypertension and stroke. BP increases start at 4 weeks of age and keeps rising until it is stabilized at about 5–6 months. Depending on the age of the animals at time of stroke and environmental conditions, mean arterial BP can vary from 120 to 200 mmHg [13]. Stroke induction leads to further increases in BP [4]. It is worth mentioning that BP is greatly affected by the depth of anesthesia and therefore the degree of anesthesia and duration of surgery should be controlled [13].

Chronic hypertension, as seen in the SHR model, results in extensive cerebrovascular remodeling of resistance vessels including reduction in lumen diameter and an increase in wall thickness and wall-to-lumen ratio [14]. These changes likely impair blood flow autoregulation and contribute to the increased infarct size after vessel occlusion. In addition, widespread astrogliosis, inflammation, and blood brain barrier (BBB) dysfunction occurs in middle age and has been implicated in the development of cognitive impairment in this model [15]. Microvascular rarefaction in numerous vascular beds, in both rat and human hypertension has been reported [16], but increased angiogenesis has also been reported in SHR [17, 18], in response to some stimuli. It is likely that the specific tissue microenvironment contributes to the pro- or anti-angiogenic state created after injury. It is unclear whether angiogenesis contributes to recovery after stroke in animals with genetic hypertension.

Advantages of using SHR in stroke studies are modeling of essential hypertension, commercial availability and the reproducibility of infarcts after distal MCAO. Disadvantages include: cost, and genetic heterogeneity depending on the colony and source [13]. SHR are also used as a model of attention deficit hyperactivity disorder [19] making them difficult to use for neurobehavioral tests that require prior training.

SHRSP are bred from SHR with stroke phenotype. They have a high incidence of spontaneous stroke which can also be modulated by the type of diet and degree of salt loading [20]. SHRSP with or without salt loading exhibit higher BP levels than SHR [20]. Although the mechanism behind the tendency of SHRSPs to develop spontaneous strokes is not clear, they may mimic small vessel disease and lacunar strokes. Interestingly, SHRSPs develop both cortical infarcts and hemorrhages and they show BBB leakage even before the development of full hypertension and vessel damage. Whether these changes are directly linked to the high blood pressure is an area of debate [21]. As they develop spontaneous strokes, SHRSP are often used in primary stroke prevention studies. However, they are also used in post-stroke intervention studies through induction of MCAO. SHR and SHRSP are more susceptible to ischemic insults than their normotensive control, the Wistar-Kyoto [20].

RHRSP were developed from the normotensive Sprague Dawley (SD) rats through insertion of clips to constrict the roots of the two renal arteries (two-kidney two-clip model). Hence, it is a model of renovascular hypertension. In comparison to other renovascular hypertension models (two-kidney one-clip in which one clip only is placed and one-kidney one-clip in which the contralateral kidney is removed), the two-kidney two-clip model showed much higher rate of spontaneous strokes (both ischemic and hemorrhagic) with an incidence of about 60% at 14 weeks [22]. The incidence of stroke appears to be dependent on the magnitude of BP increase [23]. It is presumed that the changes in the cerebral vasculature seen in this model are exclusively due to the increase in BP and independent of any genetic background. Therefore it can be used to model stroke in secondary hypertension of renal origin. While renal dysfunction is the most common cause, secondary hypertension represents less than 10% of hypertension cases and this should be taken into consideration when interpreting results generated in this model [11, 24]. Like SHRSP, this model can be used for preventive studies exploiting spontaneous strokes. It can also be subjected to MCAO which results in larger infarct size compared to their respective controls [25]. The model is inexpensive in terms of induction of hypertension if the lab has the surgical expertise but a surgical follow up period of about 12 weeks is required to achieve the expected increase in BP before induction of stroke [25].

Other models of hypertension that develop spontaneous strokes include the Dahl salt-sensitive rats. These rats were developed by Lewis K Dahl through selective breeding of rats that showed a pronounced hypertensive response upon salt ingestion. These salt sensitive rats developed moderate hypertension on normal salt diet [26]. On a high salt diet (8% NaCl), they can develop blood brain barrier disruption, stroke lesions and intracerebral hemorrhage as early as 5 weeks [27, 28]. Further increases of BP of hypertensive mice or transient acute increase of BP on top of chronic hypertension has also been described to cause spontaneous intracerebral hemorrhage. Double transgenic mice that overexpress both human renin and human angiotensinogen (a genetic model of hypertension) develop further increase in BP and intracerebral hemorrhage upon treatment with high salt + L-NAME (L-NG-Nitroarginine methyl ester, an inhibitor of nitric oxide synthase) [29]. Similarly, pharmacologically induced (angiotensin II infusion + L-NAME) chronically hypertensive mice develop intracerebral hemorrhage upon injection of angiotensin II or norepinephrine [30].

B. Translation of preclinical stroke studies in hypertensive animals to the clinic—As mentioned above, different hypertensive stroke models can model different types of strokes. Preclinical researchers should provide a thorough explanation of the hypertensive model used, including duration of hypertension, to be correctly understood by clinicians. Similarly, preclinical studies should report the age, weight, sex and BP values of the animals used. A statement about the animal age relevance to humans (e.g. using young adult or aged rats) should also be included.

Different types of hypertensive stroke patients could respond differently to treatments. Unlike patients with large infarcts due to large artery embolism, deep small vessel strokes (called lacunar strokes) are associated with long standing high blood pressure and diabetes. Moreover, patients presenting with lacunar strokes are thought to have higher BP in the

acute post-stroke period compared to those with non-lacunar strokes irrespective of the stroke severity [31]. Taking acute BP lowering intervention after stroke as an example, results from the SCAST (Scandinavian Candesartan Acute Stroke Trial) and INWEST (Intravenous Nimodipine West European Stroke) trials have shown worse outcome with acute BP lowering in patients with small infarct or lacunar strokes [32, 33]. On the contrary, acute BP reduction was shown beneficial in patients with large infarcts in the SCAST trial [32]. These patients are more prone to edema and hemorrhagic transformation (HT) and therefore could benefit from moderate blood pressure lowering. In SHR subjects subjected to transient MCAO, treatment with a hypotensive dose of candesartan showed neurovascular protection and improved recovery [4]. These animals have large infarcts that would reflect stroke in a hypertensive patient with large artery occlusion and reperfusion. In contrast, lacunar stroke patients are best modelled by SHRSPs, rather than SHRs subjected to MCAO. Therefore, choosing the best model to mimic a certain hypertensive stroke patient population is imperative.

II. Diabetes

A. Animal models of diabetes in stroke research—Diabetes increases the risk of stroke by 2 to 6 folds [34, 35]. It is important to note that hyperglycemia is an independent predictor of poor clinical outcomes after stroke. However, not all patients with hyperglycemia have a history of diabetes and some of these patients develop acute elevations in blood glucose due to multiple factors. A number of experimental models have been developed to investigate the impact of hyperglycemia on stroke outcomes and recovery. Since this review focuses on comorbidities, only models of diabetes will be discussed. Acute hyperglycemia has been recently reviewed in detail [34].

Streptozotocin (STZ)-induced Type 1 diabetes is by far the most commonly used diabetes model in stroke research. STZ is a good model of Type 1 diabetes as it targets directly the β -cells of the pancreas causing hypoinsulinemia and induces chronic hyperglycemia without other comorbidities. It can be used to induce diabetes at any age and in both sexes. However, this model has some limitations. STZ is a toxin that is taken into beta cells by the glucose transport protein, GLUT2 (glucose transporter 2), which is highly expressed in these cells rendering its toxicity relatively specific to the pancreas [36, 37]. STZ is not recognized by the other glucose transporters, however, its toxicity and proinflammatory properties have been questioned since they may impact stroke injury. Injection of non-metabolized D-glucose analogue (3-O-methyl glucose) immediately before STZ administration protects the pancreas, and animals do not develop diabetes [38, 39]. These animals do not show any signs of inflammation in the brain or in the periphery (personal unpublished data), suggesting that STZ without hyperglycemia does not have an additional effect. A single high dose STZ injection induces high blood glucose levels over 350 mg/dl, which can be prevented by the use of tapered STZ injection protocol. Unless supplemented by insulin, high blood glucose levels cause muscle wasting in long-term studies. It is also to be noted that mice are more resistant to STZ than rats, which may necessitate the use of a higher dose (100 – 150 mg/kg for mice vs 50 – 65 mg/kg for rats) or multiple doses to induce diabetes in mice. Another limitation is the lack of insulin in this model. In Type 1 diabetes, lack of

insulin may contribute to deficits observed in neuronal repair processes as insulin is known to contribute to neuronal plasticity.

In animals with high blood glucose levels, hyperglycemia leads to critical changes in the structure of the neurovascular unit. There is extracellular matrix deposition in the basement membrane of capillaries and penetrating arterioles, which leads to disruption of communication between blood vessels, astrocytic end-feet and neurons. Endothelial dysfunction also occurs early in the disease. Moreover, hyperglycemia leads to neuronal death [40]. In this model, while the infarct size depends on the severity of diabetes (Fig. 2), vascular injury (increased BBB permeability and intracerebral hemorrhage) is always exaggerated and associated with worsened functional outcomes [41–43]. Also in this model, tPA (tissue plasminogen activator) increases HT [41], as seen in patients, and the use of either minocycline [44] or early glycemic control with insulin [45] significantly ameliorates this effect. Recovery after stroke is also significantly impaired in this model [46].

Low Dose STZ and High Fat Diet (HFD) Model of Type 2 diabetes has been created to simulate the natural pathological progression of Type 2 diabetes and insulin resistance. Animals are fed with a HFD for 2 weeks and then injected with low dose STZ (30 mg/kg) [47–50]. This model develops hyperlipidemia and moderate elevations in blood glucose levels. Similar to the Goto Kakizaki (GK) model described below, there is pathological neovascularization of the brain. An ischemic brain injury overlaid on this pathology causes poorer outcomes as compared to control animals. Furthermore vascular restoration after stroke is impaired and recovery is blunted [51, 52].

GK rats are a genetic lean model of Type 2 diabetes that spontaneously develop diabetes. The model was developed from selective inbreeding of glucose intolerant Wistar rats. Therefore in most studies, Wistar rats are used as the control group. The age for onset of diabetes varies in different breeding colonies (7 to 14 weeks of age). GK rats provide a good model to study Type 2 diabetes with insulin resistance in a mild to moderate chronic hyperglycemia, as their blood glucose levels range between 160–250 mg/dl. It should be noted that hyperinsulinemia that is observed at the onset of diabetes gradually goes down as islet cells are reduced during the progression of diabetes. However, animals remain insulin resistant. In this regard, this model mimics what is seen in patients with Type 2 diabetes. While some female rats do develop diabetes at a later age, it is not typical in all female GK rats, which limits studies that incorporate sex into the experimental design. Another limitation is that it is an inbred model. Of note, when GK rats that do not become diabetic are subjected to MCAO, the neurovascular injury pattern is similar to Wistar control rats (Fig. 2).

This model presents with pathological development of extensive remodeling and pathological neovascularization of the cerebrovasculature, a finding that is common to the low dose STZ plus HFD described above. In this model, even severe ischemic brain injury (3 hours of MCA occlusion) does not increase infarct size but greatly amplifies HT 24 h post stroke resulting in poor outcomes [53–55]. When analyzed at 7 days after stroke, there is greater infarct expansion in GK rats [56]. This model also shows poor recovery that is

associated with significant vasoregression and loss of reparative angiogenesis that is typically seen in control animals after ischemic stroke [57].

Zucker diabetic fatty rats provide an experimental model of obese, type 2 diabetes that mimics the metabolic abnormalities in humans. So far it is rarely used in stroke research. In this model, there is increased leukocyte infiltration and greater infarction associated with increased cerebral expression of the inflammatory mediators and worsened functional outcomes compared to control rats [58].

The db/db mouse model of Type 2 diabetes is the most commonly used model in mice after the STZ model [39–44]. The db/db mice lack the functional leptin receptors that regulate the satiety and hunger inhibition through the action of leptin. Accordingly, these mice gain weight and develop hyper/dyslipidemia, which make them a good model to study the impact of combined comorbidities, not only diabetes, on stroke outcomes. However, blood glucose levels of db/db mice are very high (350 – 500 mg/dl) and animals are extremely obese. After stroke, diabetic db/db mice demonstrate less microglial activation as well as less and delayed expression of inflammatory mediators interleukin-1 alpha, interleukin-1 beta and tumor necrosis factor alpha, and this compromised inflammatory response is correlated with worse recovery [59]. This model exhibits increased blood brain barrier permeability after stroke that is associated with matrix metalloproteinase 9 (MMP9) activation [60]. Chen et al showed that diabetic db/db mice not only exhibited increased lesion volume, brain hemorrhage and worse neurological deficits but also showed greater white matter damage and increased MMP9 activity in the ischemic brain [61]. The ob/ob mice are similar to the db/db mouse model in general but lack functional leptin. In this model, there is also delayed and diminished initial cerebral inflammatory response and poor functional outcomes [62].

Acute reductions in cerebral blood flow result in stroke and the magnitude of the reduction predicts stroke outcomes. The development of collateral blood flow is an independent predictor of stroke outcome. Akamatsu et al showed that a robust recruitment of leptomeningeal collateral flow was detected immediately after MCAO in C57BL/6 mice, and it continued to grow over the course of 1 week. However, this phenomenon was impaired in the type 2 diabetic db/db mice and this was associated with poor functional outcome [63]. In an interesting study to assess the impact of sex difference on stroke outcomes, Vannucci et al showed that female diabetic db/db mice experienced less damage than their male counterparts when exposed to hypoxia/ischemia [64].

Other Models: There are more commercially available models of diabetes like, the OLETF (Otsuka Long Evans Tokushima Fatty) rats, a model of type 2 diabetes with obesity, transgenic RIP-HAT rats, RIP-HAT mice and GLUT 4 mice. However, these models have not been employed in stroke research.

B. Translation of preclinical stroke studies in diabetic animals—From a translational perspective, the impact of preexisting disease (severity and duration of diabetes before an ischemic event) as well as the management of diabetes on functional outcomes needs to be considered in stroke research [9, 65]. Duration of diabetes needs to be clearly reported. In addition to mean blood glucose levels measured after the onset of diabetes and

spot blood glucose levels measured at the time of stroke surgery, use of HbA1c (glycated hemoglobin) is highly recommended, as it reflects the plasma glucose concentration over a prolonged period of time.

The choice of diabetes model depends on the question being asked. If severe hyperglycemia without confounding factors is being investigated, STZ-induced diabetes can be used. It should be noted that these animals do not gain weight as their nondiabetic counterparts and animals show signs of wasting in long-term studies, which would have a significant effect on functional outcomes. Similarly, the db/db model that presents with highly elevated glucose levels, obesity and dyslipidemia would be a good model to investigate the impact of severe type 2 diabetes on stroke outcomes. However, a review of 2 pilot clinical trials that investigated the feasibility and safety of blood glucose lowering in acute ischemic stroke patients revealed important information with regard to blood glucose levels in stroke patients. Over 75% of patients enrolled in Treatment of Hyperglycemia in Ischemic Stroke (THIS) and Glucose Regulation in Acute Stroke Patients (GRASP) trials had a history of preexisting diabetes and presented with baseline median blood glucose levels in the range of 150–250 mg/dl [66, 67]. GK rats are a lean model of type 2 diabetes that exhibit insulin resistance and blood glucose levels around 200 mg/dl as seen in stroke patients. However, this model does not have any dyslipidemia or hyperlipidemia. Given that diet-induced metabolic disease is an important clinical problem, a high fat diet plus low dose STZ model may be a more suitable model of diabetes to be included in stroke research. This model is increasingly used to study complications of diabetes [47–50]. Blood glucose levels can be titrated to achieve moderate hyperglycemia and the high fat diet induces hyperlipidemia. It can be easily used in genetically engineered models. As reported under models of diabetes, pilot studies showed that sensorimotor and cognitive recovery after ischemic stroke is blunted in this model but more studies are needed.

Both stroke and diabetes are vascular diseases. Diabetes induces dysfunction and pathological remodeling of the cerebrovasculature, which ultimately affect stroke injury and recovery [68, 65]. Dysfunctional angiogenesis occurs in various models of diabetes including db/db mice, GK rats and HFD plus STZ diabetic rats [52, 69–71]. Ischemia reperfusion injury in this setting causes greater hemorrhagic transformation even at shorter occlusion times without increasing infarct size and worsens functional outcomes in GK and HFD plus STZ models. In addition, post-stroke restorative angiogenesis seen in control animals is lost in these models and this is associated with poor functional motor and cognitive recovery [72]. Thus, these models offer an opportunity to study the role of vascular injury in stroke outcomes as well as the role of vascular restoration/angiogenesis in long term recovery in diabetes.

Given the importance of the vasculature in stroke pathogenesis and recovery, it is essential to point out the differences in cerebrovascular anatomy in mouse and rat strains commonly used in stroke research (see review [73]). For example, Sprague-Dawley rats are the most commonly used animals in stroke research and they have a highly variable branching pattern of MCA, the most commonly occluded artery in stroke models [73]. Balb/c mice have extremely poor collaterals [74]. Therefore, if HFD plus STZ model is used to induce diabetes, these factors must be considered in choosing the appropriate rodent strain.

From a clinical point of view, glycemic control remains to be an important tool for prevention and treatment of diabetic complications. Regulation of blood glucose with metformin prevents dysfunctional neovascularization and cerebrovascular remodeling in diabetic GK rats [69, 53]. When these animals are subjected to ischemic stroke, vascular injury is greatly diminished and functional outcome is improved [53]. Glycemic control with metformin only in the post-stroke recovery period prevents diabetes-mediated vasoregression that occurs in the cerebrovasculature and enhances functional recovery [72]. While these findings strongly suggest that blood glucose is a critical determinant of vascular injury and recovery in diabetic stroke, it is also possible that metformin has direct effects independent of glycemic control [75]. This opens up the possibility of identifying novel targets and treatment strategies for diabetes and stroke.

III. Other Factors

A. Hyperlipidemia/Dyslipidemia/Obesity—Hyperlipidemia/dyslipidemia and obesity are risk factors for stroke that are closely linked and as such will be reviewed together in this section [76–78]. The most commonly used model for inducing hyperlipidemia/dyslipidemia and/or obesity is the administration of HFD. While composition of fat varied, most stroke studies used a cholesterol rich diet to mimic the Western diet [79–83]. Depending on the duration of diet, there is an increase in body weight and plasma lipids. In some studies, HFD is used in wild-type or apolipoprotein E (ApoE) knock-out mice to evaluate the effect of dietary and genetically induced hyperlipidemia [79–81]. HFD is known to induce insulin resistance. It needs to be emphasized that blood glucose levels and insulin sensitivity are not consistently reported in most studies. It is also recommended to report body weight, which is missing in a majority of studies that used HFD in their experimental stroke paradigm.

While some of these studies reported that HFD exacerbated neuronal injury, studies especially in the ApoE null background yielded different information. 10-week 45% HFD that results in elevated lipids, body weight and blood glucose mediates cerebrovascular remodeling and increases bleeding and infarct size [84]. Another study reported that 45% HFD for 8 weeks blunted neurovascular coupling and increased infarction and hemorrhagic transformation after stroke resulting in poor outcomes [85]. In this study, HFD increased mortality, necessitating the occlusion time to be shortened. On the other hand, western diet did not increase infarct size in other studies in wildtype mice but did so in ApoE null mice [86, 80, 81]. An interesting aspect was that hyperlipidemia attenuated the vascular endothelial growth factor (VEGF) mediated angiogenic response [81]. A common feature in all these studies is the augmentation of systemic and neuronal inflammation, a venue that must be followed for hyperlipidemia and stroke research.

While obesity is considered a risk factor for stroke, there is also increasing evidence that obesity is associated with better functional outcomes after cardiovascular events including stroke, a phenomenon known as the obesity paradox [87–90]. In experimental models in which hyperlipidemia is induced by HFD, the extent of weight gain depends on the duration of the diet but animals do not achieve the body weights observed in genetic models like db/db mice or Fatty Zucker rats. As discussed above, the db/db model is complicated by the

presence of diabetes. Lean and obese Zucker rats differ from the diabetic fatty Zucker rats and may be used for obesity research. However, in these rats, blood pressure is increased and that leads to cerebrovascular remodeling and worsened short-term outcomes after ischemic stroke [91, 92]. There are no studies that investigated long-term functional outcomes and addressed the obesity paradox in preclinical models.

IV. Comorbidities and systemic immune response

One important determinant of the stroke pathophysiology and outcome is the systemic immune response. Many studies have examined the interaction between the brain and peripheral immunity after stroke [93–96]. However, little is known about how comorbidities alter systemic immune cells function and response after stroke. The reason this has not been studied in detail could be attributed to the difficulty in dissecting the contribution of structural and functional cerebrovascular changes from immune system alteration on stroke outcome under comorbidities.

Moller et al examined the effect of hypertension on systemic immune cells in SHR after stroke [97]. To minimize the effect of cerebrovascular changes in SHR, the authors conducted a photothrombotic stroke. They found an increase in the numbers of monocytes, macrophages and granulocytes in ischemic hemispheres of SHR compared to their Wistar-Kyoto controls, which correlated with higher infarct volume at day 3 post-stroke. The increase in number of infiltrating myeloid leukocytes was attributed to the higher surface expression of the adhesion molecule, ICAM-1 (intercellular adhesion molecule 1), on these cells as well as increased expression of chemokines. We have recently reviewed the effect of diabetic stroke on the peripheral immune response [98]. Similar to hypertension, diabetes polarizes immune cells to a pro-inflammatory phenotype which was associated with worsened outcome. In hyperlipidemic mice (apoE null - fed high cholesterol diet), Herz et al reported increased post-stroke immune activation, granulocyte infiltration into the brain and pro-inflammatory response that was associated with larger infarct and worse outcome [80]. In a more recent study, they could dissect the role of systemic immune cells and establish a causative relationship with worsened stroke outcome [79]. Through neutrophil depletion or antagonizing CXCR2 (neutrophil specific receptor), the authors could reverse the exacerbated neurological deficits and infarct volumes in the hyperlipidemic ApoE null mice.

While correlational studies can shed light on the systemic immune changes in strokes with comorbidities, interventional studies that modulate the systemic immune function are needed to establish causative relationship. Such studies are very important to translate new therapeutics that target the systemic immune response to stroke patients.

CONCLUSIONS: Aging, Sex and Multi Modelling

It needs to be emphasized that most of the studies discussed above under hypertension, diabetes and hyperlipidemia/obesity were conducted in relatively young male animals and in general with one comorbid condition. It is clear that there is a need for multimodelling for successful translation of preclinical research to the clinic. Age and sex are two important non-modifiable risk factors for stroke. As recently reviewed, with aging there is a shift toward a pro-inflammatory phenotype in the brain as well as the periphery [99]. Blood brain

barrier is disrupted and ability to respond to injury is impaired. Thus, interventions to promote recovery may differ significantly.

While women are protected from stroke before menopause, females have increased stroke rates and poorer outcomes at older ages [100]. It is recognized that the use of adult reproductive female rats in stroke research is complicated by the estrous cycle. However, there is a big gap in our knowledge of stroke pathophysiology and outcomes in female disease models that must be addressed.

As demonstrated by Rewell and colleagues, stroke can be induced in aged hypertensive rats with diabetes but mortality is increased [9]. Recently described SHR/NDcp rats, which spontaneously develop obesity, hypertension, hyperlipidemia and insulin resistance, may offer unique opportunities [93]. An alternative may be the HFD + low dose STZ model, which can be modified by addition of a high salt component to the diet to increase BP. In addition, this model can be easily applied to both sexes.

In developing therapeutic targets and strategies, one also needs to acknowledge that there may be disease and treatment interactions. For example, a recent study showed that the acute inhibition of MMP-9 at reperfusion by minocycline or through inhibition of its upstream activators, peroxynitrite and NFkB, reduced MMP-9 and HT in diabetic groups. Neurological deficits were improved to varying degrees in diabetic animals receiving these acute therapies. In control animals, while all treatments reduced MMP-9 activity, bleeding was not improved. Multiple disease and drug interactions noted in this study strongly suggested that these treatments have differential effects on control and diabetic animals, and as such, therapeutic targets for neurovascular protection may differ in disease states [5]. Another study also showed that diabetic animals may actually respond better to some therapeutic interventions [6].

When all these factors are considered, we have the following recommendations for preclinical stroke studies with comorbidities.

1. Disease duration and severity need to be clearly reported with special attention to details such as, mean blood pressure in hypertension, mean blood glucose and HbA1c in diabetes studies, lipid profile and blood glucose in hyperlipidemia studies, and body weight in all of them.
2. Mortality is often higher in stroke models with preexisting co-morbid conditions, necessitating model optimization. Reduction in stroke-related mortality can often be achieved with reducing the duration of occlusion or reducing the clot size. Distal MCAO for small, cortical, survivable infarcts as well as subcortical white matter strokes, that are highly common in hypertension and diabetes, are desirable for long term recovery studies. If large artery stroke model needs to be used for comparisons for therapeutic interventions, mortality rate, which is usually doubled in comorbid disease states, must be reported.

3. When comparing stroke outcomes between controls and animals with co-morbid disease, either age OR weight must be matched. Hypertensive and diabetic animals do not gain weight at the same rate as normal healthy animals.
4. The effect of a therapeutic intervention on stroke injury and recovery can be first compared in adult male control and disease (diabetes, hypertension or hyperlipidemia) animals followed by studies in female and aged animals.
5. Estimated sample sizes should be calculated with the specific disease model planned, as variability in outcome measures is often higher than in healthy animals, leading to increased cost.

Acknowledgments

Adviye Ergul is a Research Career Scientist at the Charlie Norwood Veterans Affairs Medical Center in Augusta, Georgia. This work was supported in part by VA Merit Award (BX000347), VA Research Career Scientists Award, and NIH (R01NS083559) to Adviye Ergul; VA Merit Award (BX000891) and NIH award (NS063965) to Susan C. Fagan.

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Figure 1.

We searched PubMed using the terms 'hypertension' and 'MCAO' to screen for studies conducted in hypertensive animals using the most widely used experimental stroke model; the middle cerebral artery occlusion model. We found a total of 49 papers, 28 of them were conducted in SHRs (57%), 9 in RHRSP (19%), 6 in SHRSP (12%), and 6 in other hypertension models (12%).

Figure 2.

Glycemia level in diabetes is important for infarct size. Representative TTC images (24 h post-MCAO by suture occlusion model) from aged matched (12 weeks) male **1.** Control Wistar rats, **2.** Nondiabetic GK rats, **3.** Mild STZ diabetes in Wistar rats (induced at 5 weeks of age by tapered 20-15-10 mg/kg injection over 3 days to achieve similar blood glucose levels as in GK rats, **4.** Severe STZ diabetes in Wistar rats induced at 5 weeks of age by 65 mg/kg STZ injection), **5.** Diabetic GK rats (onset of diabetes around 5 weeks of age, and **6.** HFD plus low dose STZ- diabetes in Wistar rats (Only this group, infarct was measure at Day 3 after MCAO).

Table 1

Experimental Models of Hypertension in Stroke Research.

	HTN model			Stroke model	Outcome	Pros	Cons	References [no.]
	Induction	Severity (BP)	Duration					
SHR	Genetic	~ 180 mmHg	BP increases at 4 weeks and stabilizes at 5–6 months	Suture and embolic MCAO, Distal MCAO using photothrombotic and endothelin models	Large infarct size after MCAO Spontaneous strokes are rare	1 Modelling essential hypertension (90% of hypertensive patients) 2 Reproducible infarct size after distal MCAO 3 Readily available commercially	1 expensive 2 genetic variability based on the source 3 ADHD behavior 4 genetic differences from human essential HTN	Bouts et al, 2014 [101]; Yao et al, 2014 [102]; Emmrich et al, 2015 [103] Yao et al, 2009 [104]; Uluc et al, 2011 [105]; McCarthy et al, 2009 [106].
SHRSP	Genetic	~ 220 mmHg	at about 12 weeks of age	Stroke prevention, Suture and embolic MCAO, Distal MCAO (electrocoagulation)	Large infarct size after MCAO High incidence of spontaneous stroke - >80% at 30 weeks	1 develop spontaneous strokes 2 modelling cerebral small vessel disease and lacunar strokes		Pires et al, 2014 [107]; Kimata et al, 1991 [108]; Carswell et al, 1999 [109].
RHRSP	Induced	Peak of 200 mmHg	6–24 weeks after surgery	Stroke prevention Transient proximal and distal MCAO	Large infarct size after MCAO High incidence of spontaneous stroke - 60% at 14 weeks	1 Surgically induced, thus eliminates genetic factors 2 low cost 3 mimics secondary hypertension of renal origin	1 needs surgical manipulation followed by a wait of 12 weeks to allow for BP increase	Liao et al, 2009 [111]; Tan et al, 2014 [110]; Wang et al, 2007 [112]; Zhang et al, 2011 [113].
Dahl salt-sensitive rats	Genetic	200 mmHg	within 4 weeks on high-salt diet	Stroke prevention	Large infarct size after MCAO Develop spontaneous strokes on			von Lutterotti et al, 1992 [28].

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			high salt diet at 5 weeks				
References [no.]	Cons	Pros	Outcome	Stroke model	HTN model		
					Duration	Severity (BP)	Induction

Table 2

Experimental Models of Diabetes in Stroke Research.

HG Model			Stroke Model	Outcomes	Pros	Cons	References [no.]
Model/Induction	Severity	duration					
STZ induced diabetes	350–500 mg/dl	Chronic HG 4–6 weeks	Transient suture occlusion, Embolic occlusion	Diabetes increased infarct size, BBB permeability, HT and functional outcomes	Simulate Type 1 diabetes. Good model for diabetes without other comorbidities	Very high BG levels. Usually animals get wasted and lose weight	Reeson et al, 2015 [43]; Fan et al, 2012 [41], 2013 [44]; Ning et al 2012 [42]; Sweetnam, et al, 2015 [46].
STZ and HFD induced diabetes	200 – 300 mg/dl	Chronic HG and dyslipidemia 4–6 weeks	Transient suture occlusion, Embolic occlusion	Bigger infarcts, Increased HT, poor functional outcome and long term recovery, pathological cerebral neovascularization	Good model for Type 2 diabetes: hyperglycemia, insulin resistance and dyslipidemia		Li et al, 2013 [56]; Qu et al, 2014 [51]; Valenzaula et al, 2015 [52].
GK rats	160–250 mg/dl	Chronic HG 4–6 weeks	3 h suture occlusion/21 h reperfusion	Increased cerebral hemorrhage, pathological cerebral neovascularization	Clinically relevant BG levels. Type 2 diabetes, insulin resistance without lipid abnormalities	In bred model	Ergul et al, 2007 [55]; Elgebaly, 2010 [53]; Li et al, 2010 [54], 2013 [56]; Prakash et al, 2013 [57].
Diabetic Zucker rats	350–450 mg/dl	Chronic type 2 diabetes (8 weeks)	2 h suture occlusion/4 h reperfusion	Increased infarct size and worse functional outcomes	Good model for Type 2 diabetes: hyperglycemia, insulin resistance and dyslipidemia	Very high BG levels and extreme obesity	Ritter et al, 2011 [58].
db/db mice	350–450 mg/dl	Chronic HG and Obesity 4–6 weeks	Right common carotid ligation plus systemic hypoxia; Transient MCAO	Greater infarct associated with compromised inflammatory response, increased BBB permeability, infarct size and MMP9 expression	Good model for Type 2 diabetes: hyperglycemia, insulin resistance and dyslipidemia	Very high BG levels and extreme obesity	Kumari et al, 2007 [59], 2011 [60]; Chen et al, 2011 [61]; Akamatsu et al [63], 2015; Vannucci et al, 2001 [64].

Table 3

List of abbreviations

HTN	Hypertension
MCAO	Middle Cerebral Artery Occlusion
SHR	Spontaneous Hypertensive Rats
RHRSP	Stroke-Prone Renovascular Hypertensive Rats
SHRSP	Stroke-Prone Spontaneously Hypertensive Rats
WKY	Wistar-Kyoto
BP	Blood Pressure
BBB	Blood Brain Barrier
SD	Sprague Dawley
L-NAME	L-N ^G -Nitroarginine methyl ester
SCAST	Scandinavian Candesartan Acute Stroke Trial
INWEST	Intravenous Nimodipine West European Stroke Trial
HT	Hemorrhagic Transformation
STZ	Streptozotocin
GLUT2	Glucose Transporter 2
tPA	Tissue Plasminogen Activator
HFD	High Fat Diet
OLEFT	Otsuka Long Evans Tokushima Fatty rats
THIS	Treatment of Hyperglycemia in Ischemic Stroke Trial
GRASP	Glucose Regulation in Acute Stroke Patients Trial
ApoE	Apolipoprotein E
VEGF	Vascular Endothelial Growth Factor
ICAM 1	Intercellular Adhesion Molecule 1