Journal of Neuroscience Research 95:681–691 (2017)

Review

Sex Differences in Stroke Therapies

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Stroke is the fifth leading cause of death and acquired disability in aged populations. Women are disproportionally affected by stroke, having a higher incidence and worse outcomes than men. Numerous preclinical studies have discovered novel therapies for the treatment of stroke, but almost all of these have been shown to be unsuccessful in clinical trials. Despite known sex differences in occurrence and severity of stroke, few preclinical or clinical therapeutics take into account possible sex differences in treatment. Reanalysis of data from studies of tissue plasminogen activator (tPA), the only currently FDA-approved stroke therapy, has shown that tPA improves stroke outcomes for both sexes and also shows sexual dimorphism by more robust improvement in stroke outcome in females. Experimental evidence supports the inclusion of sex as a variable in the study of a number of novel stroke drugs and therapies, including preclinical studies of anti-inflammatory drugs (minocycline), stimulators of cell survival (insulin-like growth factor-1), and inhibitors of cell death pathways (pharmacological inhibition of poly[ADP-ribose] polymerase-1, nitric oxide production, and caspase activation) as well as in current clinical trials of stem cell therapy and cortical stimulation. Overall, study design and analysis in clinical trials as well as in preclinical studies must include both sexes equally, consider possible sex differences in the analyses, and report the differences/similarities in more systematic/structured ways to allow promising therapies for both sexes and increase stroke recovery.

Key words: stroke; sex differences; treatment; preclinical; clinical trials

Stroke is a cerebrovascular disease caused by interruption of the blood supply to the brain, resulting in rapid death of neurons and, consequently, a range of neurological problems, including loss of sensory or motor function, paralysis, depression, dementia, epilepsy, and even death. Stroke is the fifth leading cause of death and a leading cause of disability in the United States (Mozaffarian et al., 2015). Stroke can be classified broadly into two types, ischemic stroke and hemorrhagic stroke. In an ischemic stroke, a portion of the brain is deprived of blood (and therefore glucose and oxygen) resulting from a clot that obstructs blood flow or from narrowing of blood vessels. In a hemorrhagic stroke, a blood vessel ruptures, causing blood to flood into the brain, where it eventually clots. Ischemic strokes are more common (87%) compared with hemorrhagic strokes (13%); however, hemorrhagic strokes are more severe and likely to result in death.

Globally, 15 million people suffer a stroke every year; approximately 40% of these patients die, and about 30% are permanently disabled. In the United States, the incidence of stroke is higher among older individuals; 25% of all strokes occur under age 65 years, whereas 75% of all strokes occur in populations 65 years of age and older (National Center for Health Statistics, 2011). However, the biological sex of the patient is a principal variable affecting stroke incidence among aged populations. Women are more likely to have a stroke (Petrea et al., 2009), display more nonclassical stroke symptoms, and have worse stroke outcomes. Although stroke is the fourth leading cause of death overall in the United States, it is the fifth leading cause of death in men and the third leading cause of death in women (National Center for Health Statistics, 2011). In fact, the rates of stroke-related death have declined over the past 25 years for men but

SIGNIFICANCE

Stroke is the fifth leading cause of death in the United States. The incidence, age, presentation, and recovery from stroke differ between males and females. It is not surprising that current treatments for stroke have been shown to have a difference in efficacy between males and females, and research indicates that therapies that are in development or discovery should also consider differences in efficacy between the sexes. This Review outlines the current literature on therapies for ischemic stroke and specifically illustrates that sex differences in treatment efficacy should be acknowledged and incorporated in study design to improve eventual stroke outcomes.

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Received 12 April 2016; Revised 20 June 2016; Accepted 6 July 2016

Published online 7 November 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23855

not women (Roger et al., 2011). Women account for 60% of stroke-related deaths (Lloyd-Jones et al., 2010). The 5-year stroke recurrence is also disproportionately higher in females (20%) compared with males (10%) 45–64 years of age (Roger et al., 2011). Despite the observation that stroke size tends not to be different between males and females (Silva et al., 2010), a Canadian stroke registry study reported that 10% of women stroke patients were discharged to long-term care compared with 5% of men (Kapral et al., 2005). Furthermore, because women live longer than men, it is projected that stroke-related disability and institutionalization are likely to affect women more than men (Lai et al., 2005).

Sex differences in the prevalence of stroke can be attributed to both biological and sociocultural factors. In part, greater longevity among women ensures that they are overrepresented in the age groups in which strokes are common. Other factors such as outliving their spouse and/or living alone may also delay their access to health care facilities when a stroke occurs, resulting in worse outcome and poor functional recovery. This is made worse by the time limitations for current stroke therapy. The most commonly used intervention, intravenous treatment with the thrombolytic tissue plasminogen activator (tPA), has an optimal time window and is not recommended for use later than 4.5 hr after stroke onset. Therefore, delay in treatment for women with stroke can decrease their chances of receiving tPA treatment and increase the damage from an ischemic stroke.

Regardless of the reasons underlying the greater prevalence of stroke in women or the more severe outcomes, there is an urgent requirement for stroke therapies that can improve outcomes and be delivered on a broader/delayed time frame. Although sex-specific stroke therapies have not been systematically studied, existing data on preclinical and translational therapies suggest that there may be sex-specific effects of stroke neuroprotectants and therapies. This Review focuses on current research on stroke therapies, emphasizing how these therapies affect stroke outcomes in males and females. We have also included a summary of sex differences in stroke therapy in Table I. Throughout this Review, the term (biological) *sex* is used instead of *gender*. There is an emerging literature in cardiovascular medicine in which gender identity influences disease outcomes; however, in the context of stroke therapies, which is the focus of this Review, such distinctions have yet to be studied.

STROKE THERAPEUTICS: CLINICAL

Acute stroke presents a critical challenge for the emergency room physician and staff. Initially, the challenge is to identify whether the patient has had a stroke, excluding mimicking conditions such as Bell's palsy, meningitis, and diabetic confusion among others. In the case of ischemic stroke, a critical decision is whether the patient is eligible for tPA. tPA (Alteplase) is the only FDA-approved therapy for stroke, and its mode of action consists of proteolytic degradation of the clot with the goal of re-establishing circulation, known as *recanalization*. tPA has also been shown to increase the risk for hemorrhagic transformation, which occurs subsequent to ischemic stroke and cerebral infarction (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995), even in eligible patients (Katzan et al., 2000). Although hemorrhagic transformation (intracerebral hemorrhage) may occur spontaneously after ischemic stroke, thrombolytic therapy occasionally leads to this complication, possibly because of the actions of tPA on matrix metalloproteinases (Tsuruoka et al., 2014). tPA increases permeability of the blood–brain barrier in aged (18–20-month-old) compared with young (3–4-month-old) male Wistar rats and is associated with disassembly of endothelial tight junction proteins such as claudin and occludin (Kaur et al., 2011).

Sex differences in treatment among patients who receive tPA may also factor into the sex differences in stroke outcomes. In a study spanning almost a decade (1997–2006), men were more likely than women to receive intravenous (IV) tPA, angioplasty/stents, carotid endarterectomy, or cardiac reperfusion. However, toward the end of the study, sex differences in the use of IV tPA were eliminated (Towfighi et al., 2013), which suggests that greater overall tPA use and an emphasis on early time to treatment may decrease sex differences in acute stroke care. More recently, a comparison of white and black male and female stroke patients found no differences in the outcome of tPA administration in men, but reported that black women were less likely to get tPA than white women (Boehme et al., 2014) and that short-term stroke outcomes among African American women who received tPA were no different from control (Mandava et al., 2013). In a regional study, women were more likely to be excluded from tPA for hypertension compared with men, suggesting that undertreatment of stroke risk factors in women may impact stroke therapies as well (Madsen et al., 2015).

In addition to differences in administration of tPA, clinical studies have indicated that thrombolytics have differing efficacy for stroke outcomes between the sexes. Reanalysis of data from multiple clinical tPA studies (ATLANTIS, ECASS II, and CASES) has shown that women are more likely than men to have an improved 90-day outcome in response to tPA treatment (Kent et al., 2005, 2008). In other words, placebo-treated women fared significantly worse than men, but there was no difference in outcome between the sexes in the tPA groups, thereby increasing the outcome in women to a greater extent than men. This was further validated with data from the Safe Implementation of Treatments in Stroke-International Stroke Thrombolysis Register (Lorenzano et al., 2013). An additional thrombolytic, recombinant prourokinase (r-pro-UK; Prolyse) showed enhanced effects in women. In a reanalysis of the PROACT-2 clinical trial data, just as with the tPA trials, untreated females had significantly worse outcomes than untreated males, but there were no differences between males and females who received r-pro-UK (Hill et al., 2006). For patients who are less responsive to acute tPA, this sex difference may favor improvement in males instead of females (Elkind et al., 2007), which may be due

TABLE I. Summary of Sex Differences in Stroke Therapies

Therapy	Males	Females
Clinical: thrombolytics		
Tissue plasminogen activator	Improves outcome	Improves outcome; females fare worse than men without tPA administration
Recombinant prourokinase	Improves outcome	Improves outcome; females fare worse than men without r-proUK administration
Endovascular intervention	Improves outcome	Improves outcome; beneficial to both sexes independent of tPA treatment
Clinical: cortical stimulation		
rTMS	Can enhance recovery	Can enhance recovery; more effective, longer lasting inhibition in normal subjects
rTDS	Can enhance recovery	Can enhance recovery; more effective, longer lasting inhibition in normal subjects
Clinical: stem cells		
Effects of cellular sex	Different pro- and anti-inflammatory markers; effects not yet studied	Different pro- and anti-inflammatory markers; may have a higher risk of tumor formation
Transplantation	May improve recovery; sex differences not yet examined	May improve recovery; sex differences not yet examined
Preclinical: anti-inflammation		
Minocycline	Varying efficacy in human trials without examination of sex differentiation; in rodents, reduction of infarct volume may be limited to males	Varying efficacy in human trials without examination of sex differentiation; in rodents, ineffective in females both normal and ovariectomized
Preclinical: cell survival		
IGF-1	Improves stroke outcome	Improves stroke outcome; can reverse estrogenic toxicity
anti-Let7F	No effect	Improves outcome only in gonadally intact animals
Preclinical: cell death inhibition		
PARP-1 inhibition	Required for minocycline effectivity; can decrease infarct volume and ameliorate neurological deficits	Can decrease infarct volume and ameliorate neurological deficits
NO reduction	Neuroprotective	Harmful; can lead to worse outcome
Caspase inhibition	No effect	Neuroprotective

to improved recanalization rates of women in the acute phase of thrombolytic treatment (Savitz et al., 2005). However, for r-pro-UK treatment, at 2 hr posttreatment there were no differences in recanalization rates between males and females, suggesting that the difference in recanalization rates does not explain all of the improved outcomes in females in response to thrombolytics (Hill et al., 2006).

Overall, there is strong evidence that thrombolytic therapy has increased therapeutic efficacy in women compared with men. Increased assignment of tPA therapy to women should be encouraged because treatment outcomes do not differ between tPA-treated men and women, whereas, in non-tPA administered groups, males were more likely to have a better neurologic score compared with women (Shobha et al., 2010). This conclusion is also supported by data from endovascular intervention trials, in which mechanical removal of the clot was performed to re-establish circulation in large-vessel occlusion stroke. Meta-analysis of the five recent randomized trials shows that scores on the modified Rankin Scale were significantly improved for both men and women after endovascular thrombectomy and that improvement was independent of Alteplase treatment (Goyal et al., 2016). Therefore, a push to enhance treatment rates of women by either mechanical or chemical thrombectomy could greatly improve stroke outcome.

FAILED STROKE TRIALS

Although several drugs have been identified in preclinical studies, few have made it to clinical trials, and none has succeeded (Chacon et al., 2008). These include the SAINT I and SAINT II trials that tested the free radical scavenger NXY-059; the RANTTAS trials for tirilazad mesylate, a lipid peroxidation inhibitor; and the INVEST trials for the calcium channel blocker verapamil SR coadministered with the angiotensin-converting enzyme inhibitor trandolapril. Although several factors may explain why the preclinical promise of these drugs has not been borne out in clinical trials, in at least one case (tirilazad mesylate) European trials showed that the outcomes were much worse in women compared with men (Tirilazad Steering Committee, 2000). Preclinical studies with these drugs routinely failed to use clinically relevant animal models, such as the aged, or include females or those with comorbid diseases (van der Worp et al., 2005). These and other studies provided the impetus for the STAIR recommendations, which specifically included

recommendations for clinically relevant animal models (Fisher et al., 2009).

Because tPA is responsible mainly for recanalization, most preclinical studies have focused on therapies that affect the survival of perilesional tissue. In animal models, cells around the infarct can display enhanced excitability 7 days postincident (Buchkremer-Ratzmann et al., 1996; Centonze et al., 2007), which then decreases but may persist for up to 4 months after stroke (Schiene et al., 1996). The increased calcium influx into neurons from the enhanced excitability can lead to mitochondrial stress and the activation of cell death pathways (Sims and Muyderman, 2010; Szydlowska and Tymianski, 2010). Therapies to combat the excitotoxicity and restore neuronal count and function have not resulted in consistent translational promise. Although these studies have included respondents of both sexes, there has been a lack of subsequent analysis of sex differences in efficacy. Additionally, the efficacy of cortical stimulation and stem cell therapy varies with the sex of the patient and, in the case of stem cells, of the cell host. These are explored below (see Table I for summary).

Cortical Stimulation

During stroke recovery there are long-term changes to the excitatory/inhibitory ratio in the cortex. Although activity at the perilesional site can be overactive and excitotoxic, overall on the ipsilateral hemisphere there is reduced cortical activity but increased activity at distal brain regions that have circuitry to the affected region, particularly analogous structures in the contralateral cortex (Cramer, 2008). These long-term activity changes can be on the scale of months to years, with studies showing that activity changes in motor cortices in both hemispheres still occur between 2 and 4 months poststroke and that alterations to activity required for language recovery can persist for 1 year or longer (Traversa et al., 1998; Saur et al., 2006). Pharmacological intervention to adjust activity after stroke has been focused largely on the acute stroke period, but no treatment has made it through clinical trials. However, two methods of cortical stimulation, repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS), have been investigated for their ability to improve stroke recovery.

Cortical activity can be directly stimulated by rTMS or modulated by rTMS or tDCS (Fregni and Pascual-Leone, 2007). In studies with stroke patients, it has been shown that rTMS with either high-frequency stimulation to stimulate activity in the affected cortex (Khedr et al., 2005) or low-frequency stimulation to decrease activity in the contralateral cortex (Conforto et al., 2012) can enhance functional outcome in physical therapy for hemiparesis. In nonstroke subjects, tDCS has been shown to control cortical activity, and cathodal stimulation has been shown to decrease cortical activity, whereas anodal stimulation increases activity (Nitsche and Paulus, 2000). Studies with stroke patients using tDCS (similarly to rTMS) therapies in concert with physical therapy have shown that both anodal stimulation of the ipsilateral cortex and cathodal stimulation of the contralateral cortex can have therapeutic effects on motor function (Bastani and Jaberzadeh, 2012; Zimerman et al., 2012), and using these stimulation paradigms in concert can result in robust effects, with one study showing effects persisting 1 week poststimulus (Lindenberg et al., 2010).

These individual studies show the promise of cortical stimulation in stroke recovery. Overall, Cochrane Reviews have shown that there are no consistent effects of rTMS (for measurements of daily living, motor function, cognitive function, depression) or tDCS (for aphasia and naming) in comparison with sham-treated patients but suggest that more randomized control trials are required to determine fully the viability of using cortical stimulation in stroke rehabilitation (Hao et al., 2013; Pollock et al., 2014; Elsner et al., 2015). A major issue with cortical stimulation and reproducible outcomes is that many variables must be controlled to include stimulation strength, pattern of stimulation, location of electrodes, and individual variation in recovery (Gomez Palacio Schjetnan et al., 2013). Patients' biological sex has not been included in this list of variables to consider in cortical stimulation therapy, although evidence suggests that stimulus paradigms may have different effects on males and females. In a reanalysis of previous tDCS studies with healthy adult subjects, Kuo et al. (2006) showed that cathodal stimulation in the motor cortex causes greater inhibition of motor-evoked potentials in females and that the inhibition had a longer duration in females. There were no sex differences in response to anodal stimulation. Using rTMS, Gennaro et al. (2004) found similar sex differences in cortical stimulation, with enhanced inhibition of motor-evoked potentials in females. These sex differences may vary by brain region; for tDCS in the visual cortex, anodal but not cathodal stimulation showed sex differences, with females having an enhanced cortical excitation in response to visual stimulus but with no difference in the duration of effect between the sexes (Chaieb et al., 2008). These studies all examined healthy, young adults; more research examining the effects of age and disease on sex differences in cortical stimulation must be conducted. Because motor function restoration is a key therapeutic target in stroke recovery leading to enhanced independence and quality of life, the inclusion of sex as a variable in response to cortical stimulation could be valuable in elucidating appropriate paradigms for reproducible therapeutic effects.

Stem Cell Therapies

Several clinical trials are underway using stem cells to aid in tissue recovery to recover cellular loss and repair disruption to neuronal circuitry. The sourcing of the cells can be allogenic, from immortalized cells lines and cultured stem cells, or autologous, including bone marrow and adipose tissue-derived stem cells. Cells from all of these sources retain their biological sex, which could affect the outcome of therapy, and those possible differences have not been fully explored.

The use of immortalized cell lines for cell transplant has had some success in clinical trials. The NT2/D1 human embryonal carcinoma-derived cell line (ATCC CRL-1973) can be mass differentiated into neurons with retinoic acid exposure (known as LBS, NT2, or hNT neurons). These cells are safe for application for stroke therapy, and transplantation may be able to improve cognitive and motor function even years after stroke incidence (Kondziolka et al., 2000, 2005). The parent cell line for the LBS neurons was derived from a metastatic testicular tumor in a 22-year-old male; however, not considered were sex differences and changes in efficacy of the male cells in male or female patients, especially in the phase II trial in which women were a small proportion of the patients examined (28%). Additionally, these studies present a possible confounding treatment in allogeneic cell application, the use of antirejection drugs. These studies and others have used continuous cyclosporine A treatment, which may have effects of its own along with differences in metabolism between males and females (Kahan et al., 1986). The inclusion of sex as a variable in studies of *ex vivo* stem cell application both of the cell line and of the patient could help to determine whether cells have better efficacy by genetic sex and whether they provide a better outcome in patients of one sex over another.

Autologous stem cells include stem cells that occur in other regions of the body, including bone marrow and adipose tissue as well as human-induced pluripotent stem cells (hiPSCs) derived from the patient. Studies have shown that stem cells derived from males and females can differ in their pro- and anti-inflammatory markers and may vary in their propensity for particular differentiation fates (Ogawa et al., 2004; Crisostomo et al., 2007). Although autologous stem cell transplants have long been preferred because of decreased risk of rejection, recent studies have indicated that autologous transplants can also trigger immune responses, particularly induced pluripotent stem cells (iPSCs); therefore, different inflammatory markers could affect transplant stability even in an autologous transplant (Zhao et al., 2011; Charron, 2013). Inflammation can arise from iPSC transplant because of cell-to-cell variations in epigenetic markers, and iPSC expansion can result in alterations to gene expression and an induction of oncogenic potential. For example, hiPSCs from females may lose expression of X-inactive-specific transcript and have a subsequent increase in oncogene transcription, thereby increasing the risk of tumor formation as a side effect (Anguera et al., 2012). For all stem cell transplants, a rigorous screening of inflammatory markers, alteration to gene transcription, and inclusion of cellular sex as a variable will greatly enhance the chance for success with stem cell therapies.

Among the completed and published clinical trials for stem cell use, there has been a disparity in patient recruitment between males and females, only 40% (ranging from 16.7% to 56%) of patients receiving treatment being female. Most studies that recruited patients indicated that both sexes would be recruited, but, even among the larger phase II and III trials, in only one published study did the authors state their intent to examine the data to find the most appropriate target population. With the exception of LBS neurons, there is no mention of the sex of the originating cells in the clinical trials of cultured cell lines, and for allogenic cells there is no statement of control for the biological sex of the donor cells compared with the sex of the recipient. As additional clinical trials for stroke treatment move forward, the inclusion or at least reporting of sex as a variable will contribute to more efficacious treatment and enhanced recovery.

SEX DIFFERENCES IN TREATMENT EFFICACY IN PRECLINICAL STUDIES

Sex differences in stroke outcome are also well recognized in preclinical models. Specifically, young females (rats and mice) have a smaller infarct volume and better cerebral blood flow than age-matched males in both normoglycemic (Alkayed et al., 1998) and diabetic (Toung et al., 2000) animals. Young females sustain a smaller infarct compared with young males or aged female mice or rats (Selvamani et al., 2014); however, aging reverses this sex difference such that aged females show poorer stroke outcomes and significantly more mortality compared with older males (Manwani et al., 2011). Sex differences have also been noted in middle age, when adult female rats have smaller infarcts than middle-aged females (Selvamani and Sohrabji, 2010a).

The female advantage seen in animal models may be associated with either chromosomal sex or differences in gonadal hormones. In an interesting animal model called the *4-core genotype*, chromosomal contribution and gonadal contribution can be evaluated for a specific disease in XX/Sry (genetic female, gonadal male) and XY/Sry$^-$ (genetic male, gonadal female) mice (Arnold and Chen, 2009). Using this model, a recent study showed that sex differences in stroke are influenced by sex hormones and not by chromosomes (Manwani et al., 2015). This is in line with the results of a large body of experimental research showing that estrogen, the major ovarian hormone, may improve stroke outcomes. In ovariectomized females, replacement with 17β-estradiol, the inactive stereoisomer 17α-estradiol (Simpkins et al., 1997), or the conjugate equine estrogen preparation (McCullough et al., 2001) reduces infarct volume in female animals. Estrogen also reduces infarct volume in males, whereas the precursor steroid testosterone increases infarct volume in this group (Yang et al., 2002).

Although estrogen availability is likely the reason for sex differences in infarct severity in young animals, estrogen treatment is protective for both sexes at this age. For other preclinical stroke neuroprotectants, however, unexpected sex differences have been observed. Preclinical studies have focused on cell death effectors, immune modulators, and neurogenesis–angiogenesis modulators. Although most studies have used only males (usually young), several recent studies have included both males and females, and those that displayed sex differences are summarized below.

Anti-Inflammatory: Minocycline as a Case Study

Minocycline, a tetracycline antibiotic, is clinically well tolerated and has been shown to have neuroprotective effects on ischemic stroke in experimental models and clinical trials (Yrjanheikki et al., 1999; Lampl et al., 2007; Li and McCullough, 2009). It is known to cross the blood–brain barrier and, after entering the brain, can attenuate neuronal apoptosis, reduce the inflammation response by reducing microglial activation and migration of T cells, and inhibit matrix metalloproteinase-9, which remodels extracellular matrix (Yrjanheikki et al., 1999; Machado et al., 2006, 2009; Goldstein, 2008; Matsukawa et al., 2009; Fagan et al., 2011; Switzer et al., 2011, 2012; Yang et al., 2015). In experimental models of acute ischemic stroke, minocycline has shown neuroprotective effects and improved behavioral outcomes in males (Yrjanheikki et al., 1999; Alano et al., 2006; Li and McCullough, 2009).

In 2007, the first clinical trial of minocycline, an open-label, blinded-endpoint evaluation trial for acute ischemic stroke, randomly allocated 152 patients (35% female and 65% male) to a placebo or oral minocycline 200 mg daily for 5 days (Lampl et al., 2007). The study revealed that the patients treated with minocycline ($n = 74$) had significantly improved outcomes compared with the placebo group ($n = 77$; Lampl et al., 2007). In 2012, a subsequent human trial demonstrated similar beneficial outcomes in a randomized, single-blind, open-label trial of oral minocycline 200 mg daily for 5 days ($n = 23$, 43% female and 57% male) vs. control ($n = 27$, 33% female and 67% male) with acute ischemic stroke (Padma Srivastava et al., 2012). More recently, in 2013, Kohler and colleagues (2013) conducted a randomized open-label, blinded-endpoint evaluation pilot study of IV minocycline 100 mg every 12 hr for a total of five doses in acute stroke ($n = 95$; minocycline $n = 47$, 38% female and 62% male; routine care control $n = 48$, 44% female and 56% male). The study showed that IV minocycline was safe but not efficacious.

In this context, a study by Li and McCullough (2009) is particularly relevant. In C57BL/6 male and female mice subjected to middle cerebral artery occlusion (MCAO), minocycline was effective in reducing infarct volume in male mice only. Furthermore, minocycline was ineffective in ovariectomized female mice even though male and ovariectomized female mice present similar levels of estrogen. After this critical preclinical study, an open-label, evaluator-blinded clinical study of minocycline for acute stroke by Amiri-Nikpour and colleagues (2015) showed that oral minocycline 200 mg daily for 5 days was effective only in male stroke patients and not in females. In this study, which included similar numbers of females in treatment (53.8%) and control (51.9%), male patients presented with significantly lower NIH Stroke Scale score in the minocycline-treated group compared with controls, whereas no significant clinical improvement was found in female patients between groups (Amiri-Nikpour et al., 2015). The authors also reported the important point that the clinical improvement in the minocycline-treated group was significant among all patients (males and females; Amiri-Nikpour et al., 2015), indicating that study design and analyses in clinical trials as well as in preclinical studies must include both sexes equally, consider possible sex differences in the analyses, and report the differences/similarities in more systematic/structured ways to translate promising therapies to both sexes.

Cell Survival Effector: Insulin-Like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) is one of the well-known neuroprotectants for ischemic stroke in young and aging animals for both males and females. Ischemic injury is more severe in older populations compared with younger, and this difference is associated with reduced availability of insulin-like growth factor 1 (IGF-1; Selvamani and Sohrabji, 2010a). Clinical observation supports the notions that the availability of IGF-1 along with its binding protein IGFBP3 is decreased with age (Rosario, 2010) and that the ratio of IGF-1 to IGFBP3 declines faster in males than in females (Waters et al., 2003). Results from studies that used male animal models support the hypothesis that IGF-1 plays a pivotal role in maintaining brain functions in acute ischemic stroke through various mechanisms, including neuronal survival, anti-inflammation, and antithrombotic effects (Li et al., 2010; Jin et al., 2013; Patel et al., 2013). Exogenous IGF-1 treatment intranasally, intravenously, or intracerebroventricularly has been shown to decrease ischemic stroke injury (Liu et al., 2004; Rizk et al., 2007; Lioutas et al., 2015; Sohrabji, 2015). Female animal models have demonstrated that poststroke IGF-1 replacement reverses estrogen's neurotoxic effects in middle-aged ovariecotmized female rats (Selvamani and Sohrabji, 2010a,b). IGF-1 adminstered to estrogen-deficient (but not ovariectomized) middle-aged females is also capable of improving and reducing stroke-induced damage and motor impairment in the aging brain and reduces blood–brain barrier disruption and neuroinflammation (Bake et al., 2014).

It has been well established clinically that serum IGF-1 levels are positively correlated with stroke outcome. Many clinical studies have shown that lower IGF-1 levels significantly increase the risk of stroke and that higher IGF-1 levels are associated with improved stroke outcomes, suggesting that the circulating IGF-1 level may be used as a predictive value for ischemic stroke outcome (De Smedt et al., 2011; Dong et al., 2014; Tang et al., 2014) and that poststroke IGF-1 treatment may be beneficial for both sexes. However, using miRNA that regulate IGF-1, Selvamani and colleagues (2012) showed sex differences in the efficacy of this treatment and showed that intracerebroventricular anti-Let7f injection is effective only in intact females and not in males or ovariectomized females, suggesting that this miRNA action may be influenced by the hormonal milieu (Selvamani et al., 2012).

Cell Death Pathways

Poly(ADP-ribose) polymerase-1. Poly(ADP-ribose) polymerase-1 (PARP-1) activation is a major cytotoxic mechanism and plays a key role in the pathogenesis of cardiovascular and inflammatory diseases (Beneke, 2008; Peng et al., 2012; Song et al., 2013; Sun et al., 2015). Emerging data suggest that cell death pathways in ischemic stroke are sexually dimorphic (Reeves et al., 2008; Yuan et al., 2009; Liu et al., 2011; Gibson, 2013). Li and McCullough (2009) additionally showed that minocycline does not impact ischemic injuries in PARP-1-null male mice, indicating that sexually dimorphic neuroprotective effects may be attributed to PARP-1 inhibition in male mice, whereas the pathway is not affected in females (Hagberg et al., 2004; Mabley et al., 2005; McCullough et al., 2005; Lang and McCullough, 2008; Li and McCullough, 2009; Liu et al., 2011). It has been shown that the downstream pathways of PARP, including apoptosis-inducing factor and PARP polymers, also mediate cell death after ischemic insult only in males (Yuan et al., 2009).

Several studies have suggested that pharmacological inhibitors of PARP-1 differentially affect males and females in response to ischemic stroke (Mabley et al., 2005; McCullough et al., 2005). Studies using male animal models support the notion that classical (3-aminobenzamide) or selective (PJ34) PARP inhibitors reduce infarct volume and enhance long-term stroke recovery by mechanisms including suppression of the poststroke neuroinflammatory response (Takahashi et al., 1999; Couturier et al., 2003; Hamby et al., 2007). More recently, a novel water-soluble PARP-1 inhibitor, MP-124, was shown to confer neuroprotection in focal ischemia by reducing NAD depletion and PAR formation in male Sprague-Dawley rats (Fujio et al., 2009). MP-124 can provide neuroprotection in both sexes by ameliorating the neurological deficits and brain infarct volume in male and female monkeys (Matsuura et al., 2011). Multiple PARP inhibitors are currently in phase I–III clinical trials for cancer treatment (Gelmon et al., 2011; Guha, 2011; Dean et al., 2012; Wang et al., 2012); however, PARP-1 inhibitors for ischemic stroke lag behind in clinical trials (Ford and Lee, 2011).

Nitric oxide. Similarly to the PARP pathway, nitric oxide (NO) is one of the key components in a mechanism of neuronal cell death in cerebral ischemia. The mechanism includes stimulation of neuronal nitric oxide synthase (nNOS), local accumulation of NO, peroxynitrite formation and nitrosative DNA damage, and PARP-1 activation (Stagliano et al., 1997; Nemoto, 2000; Zhang et al., 2013). Accumulating evidence supports sex differences in NO synthesis levels. Forte and colleagues (1998) measured NO biosynthesis by ^{15}N-nitrate excreted in urine after the intravenous administration of L-$[^{15}N]_2$-guanidino-arginine in a healthy population. Total NO production and release were significantly higher in females (n = 11) compared with males (n = 11), suggesting sexual dimorphism of NO and its related responses.

Preclinical studies have shown that reducing NO is neuroprotective only for males and deleterious for females (McCullough et al., 2005; Yuan et al., 2009). Female nNOS-null mice exhibit worse outcome after MCAO relative to wild-type females, whereas, in the absence of nNOS, null male littermates have a better outcome compared with wild-type males, suggesting that the neurotoxicity of NO production in ischemic stroke occurs only in male mice and not in females (McCullough et al., 2005). Estrogen, present in both males and females, promotes protection of endothelial function and vascular reactivity (dilation) by enhancing endothelial NO synthase functionality and NO production and manipulating endothelium-derived hyperpolarizing factor effectivity. However, testosterone has opposing effects, increasing cerebrovascular inflammation and cerebral artery tone (Krause et al., 2006; Haast et al., 2012).

Caspases. Caspase-mediated cell death pathways also show sexual dimorphism. In females, caspase-dependent cell death pathways are initiated in the ischemic brain, whereas males tend to show a preference for PARP- and NO-dependent cell death under ischemic conditions (Siegel et al., 2010; Gibson and Attwood, 2015). Evidence indicates that using the selective pan-caspase inhibitor quinoline-Val-Asp(Ome)-CH2-O-phenoxy (Q-VD-OPh) results in neuroprotective effects in both neonatal and adult female mouse postischemic stroke but results in no effects in males, indicating intrinsic sensitivity to caspase-mediated cell death mechanism in females (Renolleau et al., 2007). Additional evidence from Liu and colleagues (2009) has shown an early release of cytochrome C and an increased caspase activation after stroke in female mice but not in males and that Q-VD-OPh-treated female C57BL/6 mice have smaller infarcts and better neurological outcome, unlike male mice, in which neuroprotection is not observed, suggesting that caspase inhibition is beneficial mainly in females. Although caspase pathways are activated in both sexes after stroke, regulating this pathway promotes neuroprotection in females but not in males. In contrast to pan-caspase inhibitor, caspase 9-specific inhibitor delivered intranasally spares brain damage and improves neurological outcome poststroke in male mice and rats.

Overall, evidence is accumulating that stroke-induced cell death pathways are sexually dimorphic; PARP- and NO-mediated cell death pathways predominant in males, whereas caspase- and mitochondrial cytochrome c-dependent cell death pathways predominate in females (Table I). Therefore, a push to include both sexes in preclinical research could greatly benefit clinical stroke trials.

CONCLUSIONS

It has been observed for decades that the preclinical promise of many drugs for stroke has not successfully translated to clinical trials. One of the major reasons for failed stroke trials may stem from the exclusion of both

sexes in preclinical studies and failure to stratify by sex in clinical trials. Even though aged women have a higher risk for stroke, worse outcomes, and poorer recovery after the insult compared with aged men, preclinical studies have routinely failed to use clinically relevant animal models (e.g., aged female model) and ignored sexual dimorphisms in underlying mechanisms. In addition, evidence from primary prevention studies that estrogen therapy may increase ischemic stroke and evidence from secondary prevention studies that estrogen therapy may increase mortality (for review see Hurn and Brass, 2003) suggest that hormone use among women enrolled in clinical stroke trials should also be included. To translate promising preclinical therapies to the bedside, scientists and clinicians must consider sex as a critical biological variable in research design and analysis, report the outcomes in more structured ways, and be cognizant of the fact that therapies may be sex specific.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors participated equally in the writing of this Review.

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