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Enhanced Apoptosis From Early Physical Exercise Rehabilitation Following Ischemic Stroke

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The effectiveness of the rehabilitative benefits of physical exercise appears to be contingent upon when the exercise is initiated after stroke. The present study assessed the hypothesis that very early exercise increases the extent of apoptotic cell death via increased expression of proapoptotic proteins in a rat stroke model. Adult male Sprague-Dawley rats were subjected to middle cerebral artery occlusion (MCAO) for 2 hr using an intraluminal filament and assigned to four nonexercise and three exercise groups. Exercise on a Rota-Rod was initiated for 30 min at 6 hr (considered very early), at 24 hr (early), and at 3 days (relatively late) after reperfusion. At 24 hr after exercise, apoptotic cell death was determined. At 3 and 24 hr after exercise, the expression of pro- and antiapoptotic proteins was evaluated through Western blotting. As expected, ischemic stroke significantly increased the levels of apoptotic cell death. Compared with the stroke group without exercise, apoptotic cell death was further increased ($P < 0.05$) at 6 hr but not at 24 hr or 3 days with exercise. This exacerbated cell injury was associated with increased expression of proapoptotic proteins (BAX and caspase-3). The expression of Bcl-2, an antiapoptotic protein, was not affected by exercise. In ischemic stroke, apoptotic cell death was enhanced by very early exercise in association with increased expression of proapoptotic proteins. These results shed light on the time-sensitive effect of exercise in poststroke rehabilitation.

Key words: ischemia/reperfusion injury; rehabilitation; apoptosis; BAX; caspase-3; BcL-2; AB_2243455; AB_637828; AB_2227995; AB_631746; AB_631736

Postinjury exercise therapy that aims to ameliorate physical disability after stroke has long been considered a logical candidate for neuroprotective rehabilitation (Arya et al., 2011). In previous studies, neuroprotection has been consistently evidenced by physical exercise through reduced sequelae of brain infarction with improved functional outcomes in a rat ischemic stroke model (Ding et al., 2006; Dornbos et al., 2013; Q.W. Zhang et al., 2013). With experimental animal models of stroke, some studies have suggested a beneficial effect of exercise initiated as early as 24 hr after the onset of ischemic or

SIGNIFICANCE

The use of exercise-mediated adaptations to attenuate physical disability after stroke is an emerging arena in neurotherapeutics. However, fundamental questions regarding initiation time, which affect rehabilitation, remain unanswered. Although current guidelines recommend starting out-of-bed activity "early" during the acute phase of care, such guidelines do not specify how early exercise optimizes outcome. Our results shed light on the time-sensitive effect of exercise in poststroke rehabilitation.

F. Li and W. Shi contributed equally to this work.

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hemorrhagic stroke (Park et al., 2010; Matsuda et al., 2011; P. Zhang et al., 2013). Furthermore, only mild to moderate but not heavy exercise, if initiated early, is thought to promote recovery from ischemic stroke in rats (Lee et al., 2009). In contrast, training initiated 24 hr after permanent focal brain ischemia was found to exacerbate cortical tissue loss (Humm et al., 1998; Risedal et al., 1999). Furthermore, increased injury was detected in the forelimb area within the sensorimotor cortex of rats that were forced to overuse the impaired forelimb for 7 or 15 days postischemic stroke (Kozlowski et al., 1996; Humm et al., 1998). Together these studies highlight the importance of exercise timing poststroke with regard to recovery from injury and physical disability.

The molecular underpinnings of apoptotic cell death following cerebral ischemia are well established. During periods of reduced oxygen delivery, proapoptotic proteins, such as caspase-3 and BAX, become upregulated and are one of the major causes of neuronal death during ischemia/reperfusion injury (Wu et al., 2003). Conversely, Bcl-2 is an antiapoptotic protein that plays a critical role in cellular survival by acting as a repressor of apoptosis (Korsmeyer, 1995). Thus, the aim of the present study was to determine the effect of physical exercise therapy on apoptotic cell death and the expression of associated pro- and antiapoptotic proteins. We directly compared the effect of poststroke exercise on brain injury at 6 hr, 24 hr, and 3 days after reperfusion with the corresponding nonexercise group. Following a 2-hr middle cerebral artery occlusion (MCAO), we evaluated the extent of apoptotic cell death and the expression of proapoptotic (caspase-3 and BAX) and antiapoptotic (Bcl-2) proteins.

MATERIALS AND METHODS

In total, 56 adult male Sprague-Dawley rats (280–300 g; Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were used in this study. The protocol was approved by the Animal Care and Use Committee, Capital Medical University, Beijing, China. This study was conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Careful measures were undertaken to minimize animal suffering and reduce the number of animals sacrificed. Adult male Sprague-Dawley rats (RRID:RGD_734476) were housed in standard laboratory cages and left undisturbed for 3 days after arrival. Animals could freely walk to water and laboratory chow and were maintained in a temperature- and humidity-controlled room on a 12:12-hr light/dark cycle with light onset at 7:00 AM. The rats were randomly divided into seven groups, including a normal control group, three (6 hr, 24 hr, and 3 days of reperfusion) stroke groups without exercise, and three stroke groups with exercise that was initiated at 6 hr, 24 hr, and 3 days after reperfusion. The rectal temperature was maintained from 36.5 °C to 37.5 °C using a circulating heating pad and a heating lamp.

Focal Cerebral Ischemia

This model has been described previously (Wang et al., 2012). Briefly, male Sprague-Dawley rats were initially anesthetized in a chamber with 1–3% isoflurane along with a mixture of 70% nitrous oxide and 30% oxygen. The rats were transferred to a surgical table, and the anesthesia was maintained with a facemask using 1% isoflurane delivered from a calibrated precision vaporizer. Poly-L-lysine-coated intraluminal nylon (4.0) sutures were used to yield consistent infarcts and greatly reduce interanimal variability.

Rota-Rod Exercise

The Rota-Rod (R03-1; XinRuan Instruments, Inc., Shanghai, China), which involves motor balance and coordination training, was used as the training procedure in this study. The rod is 7 cm in diameter and 11 cm in length and is covered with smooth rubber. Animals were required to run at 35 rpm for a total of 30 min running on the rod. The apparatus delivered an electric shock to animals that dropped, thus stimulating them to stay on the rod and exercise continuously for the entire time. Nonexercise controls and exercised animals were housed in groups of three in standard cages for an equal amount of time.

Apoptotic Cell Death Detection by ELISA

For quantification of apoptosis-related DNA fragmentation, a commercial enzyme immunoassay was used to determine cytoplasmic histone-associated DNA fragments (Cell Death Detection ELISA; Roche Diagnostics, Indianapolis, IN; No.11774425001) as we described previously (Fu et al., 2013). The degree of apoptosis was quantified by the amount of cytoplasmic histone-associated DNA fragments in the sham-operated group and variable groups (stroke, stroke + exercise initiated at 6 hr after reperfusion, stroke + exercise at 24 hr, or stroke + exercise at 3 days). Apoptotic cell death was measured at 24 hr after physical exercise.

Protein Expression

Western blot analysis was used to detect protein expression in the ischemic tissue, as described previously (Wang et al., 2012). Upon conclusion of electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane. Membranes were incubated with a primary antibody (rabbit polyclonal anti-Bcl-2, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA; catalog No. sc-783, RRID:AB_2243455; or rabbit polyclonal anti-cleaved caspase-3 antibody, 1:5,000, Santa Cruz Biotechnology; catalog No. sc-7148, RRID:AB_637828; or rabbit polyclonal anti-BAX antibody, 1:500; Santa Cruz Biotechnology; catalog No. sc-493, RRID:AB_2227995) for 24 hr at 4 °C. Next, membranes were washed three times with PBS for 6 min each and reincubated with a secondary antibody (goat anti-rabbit IgG, Santa Cruz Biotechnology; catalog No. sc-2004, RRID:AB_631746; or goat anti-mouse IgG, Santa Cruz Biotechnology; catalog No. sc-2005, RRID:AB_631736) for 1 hr at room temperature. An ECL system was used to detect immunoreactive bands by luminescence. Western blot images for each antibody, including β-actin, were analyzed in an image analysis program (ImageJ 1.42; National Institutes of Health) to quantify protein expression in terms of relative image density. The mean amount of protein expression from the control group after stroke was assigned a value of 1 to serve as

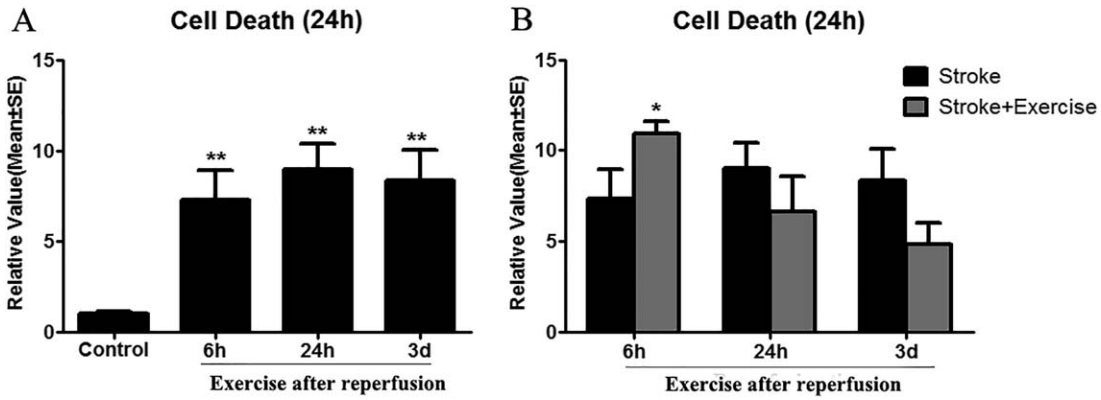

Fig. 1. **A**: Apoptotic cell death detected via ELISA was measured 24 hr after exercise termination or the equivalent time point. Compared with sham-operation controls, cell death was significantly elevated ($F_{7,28} = 8.152$, $n = 8$, $^{**}P < 0.01$) in all nonexercise ischemic rat groups (6 hr, 24 hr, and 3 day). One-way ANOVA of nonexercise groups indicates significant difference between sham-operation groups compared with ischemic rat groups ($^{**}P < 0.01$). **B**: In contrast to the nonexercise groups, very early exercise at 6 hr largely ($^{*}P < 0.05$) increased cell death, although the later exercise groups conducted at 24 hr and 3 days after reperfusion demonstrated a reduction in apoptosis, though not to a significant degree. t-test of 6 hr data indicates significant difference between exercise group compared with nonexercise group ($^{*}P < 0.05$).

reference. The expression of pro- and antiapoptotic proteins, which precedes apoptosis, was measured at 3 and 24 hr after exercise to track increases or decreases in protein expression during this time window.

Statistical Analysis

All data are mean ± SE. Cell death among multiple groups without exercise was assessed using one-way analysis of variance (ANOVA) with a significance level at $P < 0.05$ (SPSS software version 17; SPSS Inc., Cary, NC). Post hoc comparison between groups, without corrections, was achieved by using the least significant difference (LSD) method. Cell death, BAX protein expression, caspase-3 protein expression, Bcl-2 protein expression, and BAX/Bcl-2 ratio between exercise and nonexercise groups at 3 hr, 24 hr, and 3 days were compared statistically by t-test.

RESULTS

Apoptotic Cell Death

To study whether exercise timing contributes to brain injury after stroke, the degree of apoptotic cell death was measured (Fig. 1). Rats were sacrificed 24 hr after undergoing or not undegoing three different exercise regimens. Compared with control (no stroke), cell death was significantly elevated by ischemia/reperfusion injury in the three nonexercise groups (6 hr, 24 hr, and 3 days of reperfusion; Fig. 1A). This increased cell death was significantly enhanced in the very early exercise group (at 6 hr) but not in the later (24 hr and 3 days) exercise groups, in which a decrease in cell death was seen (Fig. 1B).

Proapoptotic Protein Expression

The protein expression at two time points (3 hr and 24 hr) after exercise was measured by using Western blot. Rats were sacrificed at 3 hr and 24 hr following exercise or the equivalent time point in the nonexercise group. The expression of proapoptotic proteins caspase-3 and BAX measured at 3 and 24 hr after exercise or equivalent time periods are shown in Figures 2 and 3, respectively. In the very early exercise group (at 6 hr), a significant increase ($P < 0.05$) in BAX expression was seen at both 3 and 24 hr, but not in the later (24 hr and 3 days) exercise groups (Fig. 2). Similarly, a slight increase in cleaved caspase-3 expression was seen in the very early exercise (6 hr) group at both 3 and 24 hr compared with the nonexercise group, although some increases did not reach a significant level (Fig. 3). Western blot analyses also demonstrated reduced caspase-3 protein expression in late exercise (3 days) group at both time points after exercise (Fig. 3). These findings indicate that late exercise may exert an inhibitory effect on proapoptotic protein expression and that very early exercise may enhance proapoptotic protein expression.

Antiapoptotic Protein Expression

Compared with the nonexercise group, no change in Bcl-2 protein expression was seen with very early exercise (6 hr) at 3 and 24 hr after exercise or equivalent time periods in ischemic rats. Exercise (24 hr) decreased ($P < 0.05$) the expression of Bcl-2 at 3 hr after exercise but increased ($P < 0.01$) Bcl-2 expression at 24 hr after exercise. There was a slight reduction in Bcl-2 protein expression in the late exercise (3 day) group at 3 and 24 hr after exercise (Fig. 4).

Ratio of BAX/Bcl-2 Protein Expression

The interplay between proapoptotic (BAX) and antiapoptotic (Bcl-2) expression was examined by measuring the BAX/Bcl-2 ratio at 3 and 24 hr after exercise in the very early exercise (6 hr), early exercise (24 hr), and late exercise (3 day) groups. At 3 and 24 hr after exercise or the equivalent time points, the BAX/Bcl-2 ratio was significantly

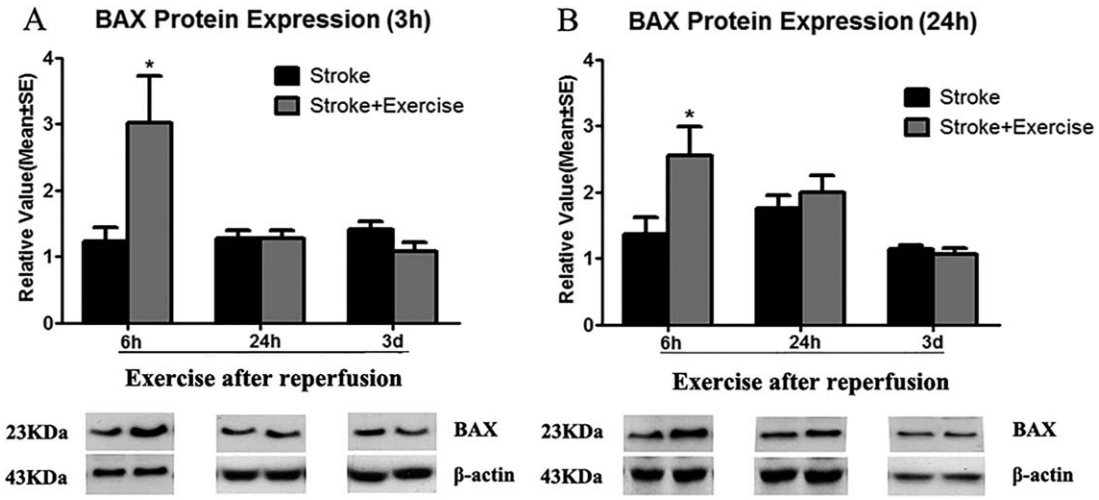

Fig. 2. **A**: BAX protein expression at 3 hr after exercise termination was significantly ($\star P < 0.05$) increased compared with nonexercise ischemic rat groups at 6 hr after reperfusion. In contrast, a slight decrease in BAX expression was seen with exercise at 3 days. **B**: A significant increase in BAX protein expression at 24 hr after exercise termination ($\star P < 0.05$) was also observed in the early (6 hr) exercise group, although a reduction was seen in the late exercise groups. Representative immunoblots are presented. t-test of 6 hr data indicates significant difference between exercise group compared with nonexercise group ($\star P < 0.05$).

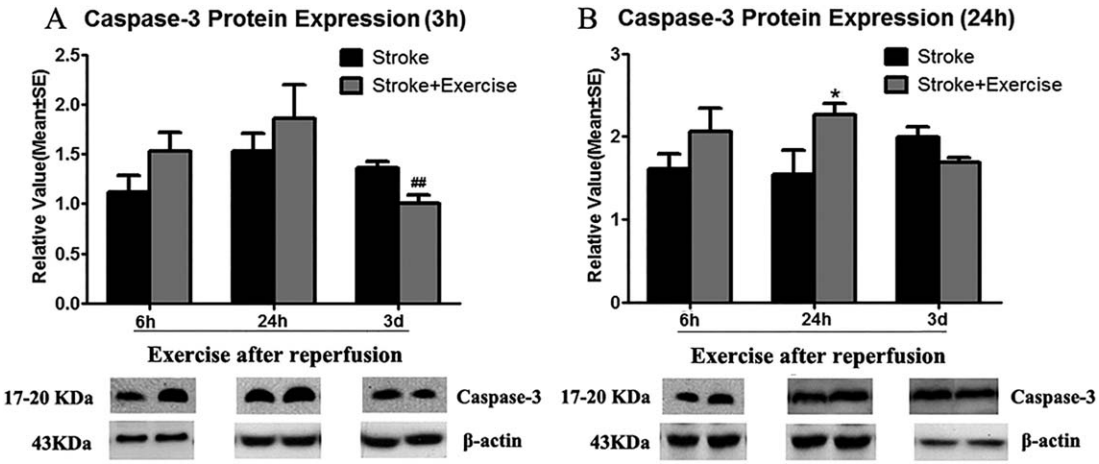

Fig. 3. **A**: Cleaved caspase-3 protein expression was measured 3 hr after exercise termination or the equivalent period. Early exercise (6 hr and 24 hr) resulted in increased caspase-3 protein expression compared with nonexercise ischemic rat groups, although a slight decrease was seen with late exercise (3 days). **B**: For 24 hr after exercise termination, similar results were seen in which early exercise (6 and 24 hr) resulted in an increase in caspase-3 protein expression, although late exercise (3 days) resulted in a decrease in caspase-3 expression. Representative immunoblots are presented. ⋆t-test and ##t-test indicate significant difference between exercise group compared with nonexercise group ($P < 0.05$).

elevated ($P < 0.05$) with very early initiation of exercise at 6 hr compared with nonexercise. No significant difference in the BAX/Bcl-2 ratio between exercise and nonexercise was observed in the other exercise groups (Fig. 5).

DISCUSSION

Accumulating evidence supports the notion that physical exercise after stroke might improve functional outcome by inducing neuronal plasticity. Previous laboratory studies using rat models have implemented exercise as early as 24–48 hr postischemia (Lee et al., 2009; Matsuda et al., 2011), and chosen 4 or 5 days for initiating relatively late exercise (Ding et al., 2003, 2004b; Tamakoshi et al., 2014). The earliest phase of exercise implementation in human clinical trials (AVERT) is within 24 hr (median time of 18.5 hr) after stroke (Dite et al., 2015). Comparable age data between rat and human suggest that 24 hr for

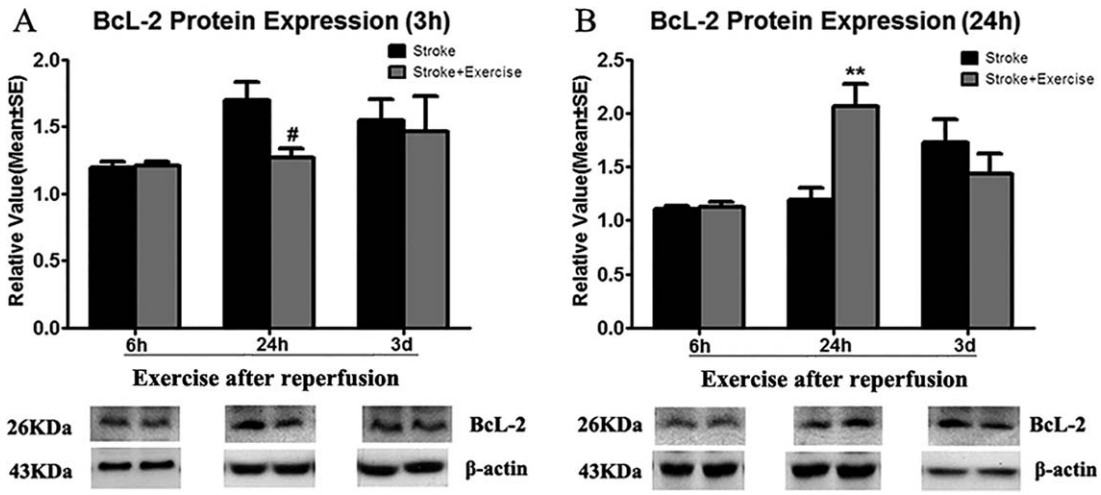

Fig. 4. Bcl-2 expression at both 3 hr (**A**) and 24 hr (**B**) after exercise termination was similar to that of the nonexercise ischemic rat group after the 6 hr equivalent time point. Bcl-2 expression was significantly decreased 3 hr after termination of exercise in the early exercise group (24 hr; $^{\#}P < 0.05$; A) but was increased at 24 hr ($^{\star\star}P < 0.01$; B). Representative immunoblots are presented. $^{\#}$t-test and **t-test of 24 hr data indicate significant difference between exercise group compared with nonexercise group ($P < 0.05$).

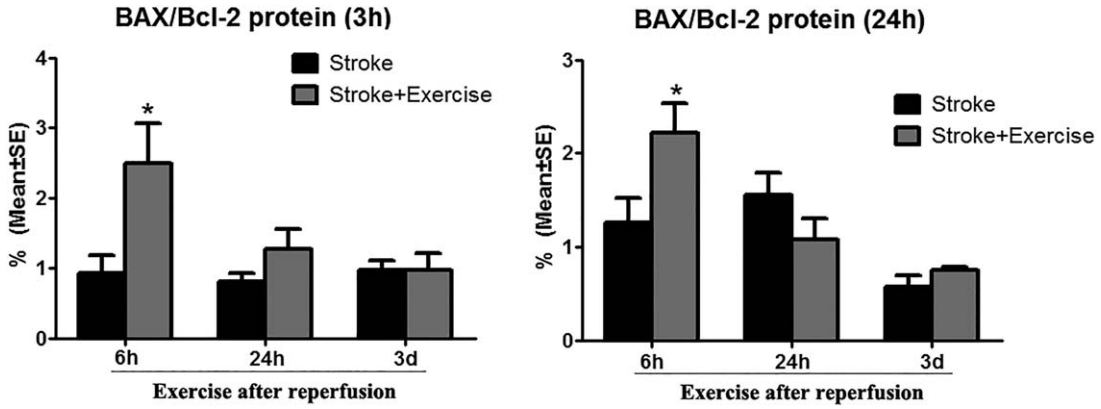

Fig. 5. At 3 hr (**A**) and 24 hr (**B**) after exercise or equivalent time period, the BAX/Bcl-2 ratio was significantly elevated ($P < 0.05$) with very early initiation of exercise at 6 hr compared with nonexercise ischemic rats. No significant difference in the BAX/Bcl-2 ratio between exercise and nonexercise was observed in other groups (24 hr and 3 day of reperfusion). t-test of 6 hr data indicates significant difference between exercise group compared with nonexercise group ($^{\star}P < 0.05$).

an adult rat corresponds to ~31 days for an adult human (Sengupta, 2013). Although this correlation may be imperfect, it raises questions about whether exercise implementation at 24 hr in rats is too late when translated to a rehabilitative strategy in humans. Therefore, we opted to use three postreperfusion exercise initiation time points (6 hr, 24 hr, and 3 days). Although 6 hr might be very early for clinical treatment in humans, this time point maybe more appropriate for studying poststroke exercise in the rat model based on differences in life span. Conversely, 3 days was chosen to determine the effect of very late exercise initiation. To our knowledge, this is the first study to use a spectrum of times to evaluate accurately the optimal timing for exercise initiation. Although this study did not clearly suggest a conclusive time point at which to establish a rehabilitative exercise strategy in humans, which will be the focus of our future study, we suggest here that very early exercise implementation is detrimental, whereas relatively late exercise implementation is less damaging.

The present study sought to determine the time-sensitive effect of exercise on apoptotic cell death after ischemic stroke and the expression of pro- and antiapoptotic proteins. The study revealed that very early (6 hr) poststroke exercise induced a significant increase in apoptotic cell death, although a possible trend of reduced

apoptotic cell death was observed with early (24 hr) and late (3 day) exercise. The present data are in agreement with our unpublished data on brain infarct volume and functional outcome exacerbated by physical exercise at 6 hr. Furthermore, we have demonstrated in previous studies that enhanced neuronal apoptosis seen with early exercise initiation (at 6 hr) postischemic stroke was associated with significantly increased production of reactive oxygen species (ROS) and lactate, although ATP levels and NADH activity were significantly reduced compared with nonexercise (data not shown). Finally, our present findings are consistent with the conclusions of a recent systematic review article that recognized a short therapeutic time window to initiate exercise, with exercise onset before 3 hr or beyond 3 days offering little benefit (Austin et al., 2014). Thus, very early exercise initiated 6 hr after stroke may be detrimental by exacerbating neuronal apoptosis, although late exercise at 3 days appears to be beneficial through a reduction in neuronal apoptosis.

Apoptosis is a major process that occurs after transient cerebral ischemia and is regulated by the pro- and antiapoptotic proteins of the Bcl-2 family. Antiapoptotic Bcl-2 members include Bcl-2, whereas proapoptotic members include BAX (Mayer and Oberbauer, 2003; Lazou et al., 2006). Upregulation of Bcl-2 with a concurrent decrease in BAX/Bcl-2 ratios appears to play a key role in protective preconditioning against lethal ischemic injury in brain and heart tissue (Wu et al., 2003; Lazou et al., 2006; Rybnikova et al., 2006). Bcl-2 also serves as a regulator of another group of apoptosis-regulatory proteins, the caspases. Caspase-3 is an aspartate-specific cysteine protease that serves as an important executioner in cell death (Fan et al., 2005). Thus, elevation of Bcl-2 combined with the downregulation of proapoptotic factors such as caspase-3 and BAX may contribute to decreased neuronal cell death following ischemic stroke. In the present study, a significant increase in BAX expression was seen with very early exercise initiation (at 6 hr) but not with the later 24-hr and 3-day exercise time points. Caspase-3 expression increased slightly in a similar fashion with very early exercise (at 6 hr) compared with nonexercise. Although caspase-3 expression at 3 hr after exercise led to a slight increase in the early exercise group (at 24 hr), a significant increase was observed at 24 hr after exercise. With the initiation of late exercise (at 3 days), a significant reduction in caspase-3 protein expression at 3 hr after exercise and a slight decrease at 24 hr after exercise were observed. In comparison with nonexercise, no change in Bcl-2 expression was seen with very early exercise (at 6 hr). Early exercise (at 24 hr) displayed mixed results, with a significant decrease in Bcl-2 expression observed at 3 hr after exercise, whereas, in contrast, a significant increase in Bcl-2 expression was observed at 24 hr after exercise. Finally, a slight but insignificant reduction in Bcl-2 protein expression was seen with late exercise (at 3 days). These results suggest very early exercise (at 6 hr) postischemic stroke may aggravate neuronal cell death by increasing proapoptotic expression (BAX and caspase-3) rather than via downregulation of antiapoptotic expression (Bcl-2).

With the initiation of early exercise (at 24 hr), the picture is less clear. Although caspase-3 expression measured 3 hr after exercise was slightly elevated, Bcl-2 expression measured 3 hr after exercise was significantly decreased. On the other hand, caspase-3 and Bcl-2 expression measure 24 hr after exercise were both significantly elevated. These conflicting findings may serve to highlight the importance of exercise initiation at 24 hr poststroke as a critical time point. During this time period, the introduction of exercise exerts a neuroprotective effect by stimulating Bcl-2 protein expression following exercise cessation over a delayed time course from 3 hr, when Bcl-2 is significantly depressed to 24hr where Bcl-2 is significantly elevated. However, this benefit of exercise at 24 hr poststroke must be weighed against the significantly increased caspase-3 expression that occurs from 3 to 24 hr following exercise cessation. Finally, the possible trend of reduced apoptotic cell death observed with late exercise initiation (at 3 days) following stroke may be explained by the downregulation of caspase-3 expression seen 3 hr after exercise cessation.

In comparison with treadmill running, the Rota-Rod has been shown to be a superior method for assessing balance and coordination, both factors that can be affected by stroke (Seo et al., 2010). Additionally, our previous results have indicated that Rota-Rod training, with similar or less intensity compared with simple treadmill exercise, results in improved functional outcomes, as evaluated with a series of motor tests (foot fault placing, parallel bar crossing, rope and ladder climbing; Ding et al., 2002). The electric shock provided to rats that fell from the Rota-Rod may have induced a stress response, potentially confounding our results and overstating the effect of early exercise. The influence of electric shock on cerebral infarct volumes has been addressed in our previous work (Hayes et al., 2008), indicating that forced exercise (exercise + shock) groups exhibited smaller infarct volumes than voluntary exercise groups and groups that were exposed only to electric shock. Furthermore, a 2014 systematic review concluded that forced exercise consistently reduces lesion volumes and is protective against oxidative damage and inflammation (Austin et al., 2014). Together these results suggest that the electric shock employed in this study likely had a minimal role in exacerbating the effect of very early exercise on postischemic brain injury.

Exercise training has been considered a means of offsetting age-induced physiological and anatomical alterations in heart and brain (Abete et al., 2000; Colcombe et al., 2004; Navarro et al., 2004). It has been demonstrated that exercise training restores and improves plasticity in the aging brain and serves to reduce both biological and cognitive senescence (Colcombe et al., 2004; Navarro et al., 2004). It has been well documented that physical activity increases blood vessel density in the adult brain (Black et al., 1990; Isaacs et al., 1992; Swain et al., 2003; Ding et al., 2004a,b). Exercise training may play a role in the

expression of angiogenic factors in aging rats (Ding et al., 2004b), whereas, for adult rats, exercise training appears to play a role in the regulation of apoptotic cell death.

In summary, this study demonstrates that increased expression of proapoptotic proteins such as BAX and caspase-3 seen with very early exercise initiated 6 hr post-stroke may underlie apoptotic cell death. The data also support an initial timing window of 24 hr to 3 days in order to enhance exercise's benefits and avoid potential detriments that may hamper better outcome. The present results may lead to the development of an effective stroke rehabilitation strategy for improved functional outcome beyond the currently achieved levels.

CONFLICT OF INTEREST STATEMENT

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial or nonfinancial interests in the subject matter or materials discussed in this article.

ROLE OF AUTHORS

FL and WS were involved with experiment design, performance, data analysis, and writing the article. EZ, XL, CP, JS, and SW were involved with performance of experiments and data analysis. XG and YD participated in experiment design, data analysis, and writing and revising the article.

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