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Efficacy of Combined Atorvastatin and Sildenafil in Promoting Recovery after Ischemic Stroke in Mice

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Abstract

Objective—The aim of this study was to test the hypothesis that a combination of atorvastatin and sildenafil promotes recovery in an additive manner after ischemic stroke in mice.

Design—Adult C57BL/6 mice (n=67) were subjected to transient middle cerebral artery occlusion. Vehicle-control (H₂O), atorvastatin (0.3mg/kg), sildenafil (0.3mg/kg), or combined atorvastatin (0.3mg/kg) and sildenafil (0.3mg/kg) were administered via oral gavage daily for 6 days starting 24 hours after ischemia. Behavioral studies including neurological score and adhesive removal test were performed before surgery and on postoperative days 1 and 7; cylinder test was performed before surgery and on postoperative day 7. Mice were sacrificed after 7 days and brain slices were stained with Haematoxylin & Eosin to measure the infarct volume.

Results—The combination group performed significantly better in the adhesive removal test (average $\pm$ SD) (50 ± 54 seconds) as compared to the control group (147 ± 109 seconds) ($P < 0.05$) and to atorvastatin (144 ± 102 seconds) ($p < 0.05$), but did not show statistically significant improvement as compared to sildenafil (107 ± 115 seconds) ($p = 0.148$). There were no significant differences among the groups in neurological score and cylinder test. There was no significant difference in the infarct volume.

Conclusions—The data suggest that combined atorvastatin and sildenafil generates a better functional outcome as compared to atorvastatin-only treatment, but not sildenafil-only treatment, in one of multiple variables tested.

Keywords

Stroke; Atorvastatin; Sildenafil; Recovery; Angiogenesis

Stroke is the third-leading cause of death in the United States and the first-leading cause of disability in the adult population. However, there are few effective therapies to promote post-acute recovery despite the fact that numerous compounds were tested clinically.

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Statins are widely used to prevent ischemic stroke and have been found to possess neurorestorative properties¹ via pleiotropic mechanisms.² In rodents, statins induce angiogenesis and neurogenesis after stroke, and improve functional recovery.^{3,4} The effect of statins on angiogenesis and neurogenesis has been shown in different disease models. Pitavastatin increased angiogenesis in a hindlimb ischemia model via Notch1.⁵ Statins have also been shown to improve neurogenesis after traumatic brain injury⁶. The mechanisms of action of statins involve many factors, including upregulating endothelial nitric oxide synthase (eNOS)^{3,7}, increasing nitric oxide (NO) bioavailability⁸, increasing cerebral blood flow⁷, improving endothelial dysfunction⁹, and regulating progenitor cells.¹⁰ Although statins are promising for promoting stroke recovery, clinical trials of atorvastatin¹¹ and simvastatin¹² in the treatment of post-acute ischemic stroke failed to show improvement in functional status.

We hypothesized that adding another agent with a synergistic effect with statins would potentiate the effect in promoting recovery. Phosphodiesterase-5 inhibitors (PDE5i) (e.g. sildenafil, vardenafil, and tadalafil) are another class of neurorestorative drugs that modulate NO signaling. They inhibit cyclic guanosine monophosphate (cGMP); cGMP hydrolysis, thereby elevating cGMP levels. Sildenafil was found to increase cGMP, and promote neurogenesis and motor recovery in stroke rats^{13,14}. However, a study of facial nerve crush injury in rodents failed to show neuroprotection by sildenafil.¹⁵ The conflicting data suggest that sildenafil alone might not be a potent neuroprotective agent. On the other hand, sildenafil has been shown to produce a synergistic effect with statins in the cardiovascular system via the NO pathway.^{16,17} Furthermore, pretreatment with combined simvastatin and dipyridamole, a phosphodiesterase 5 inhibitor, improved cerebral blood flow after stroke via the NO pathway.¹⁸ However, it is not clear if a combination of statin with a phosphodiesterase-5 inhibitor promotes long-term recovery after cerebral ischemia.

In this study, we tested the hypothesis that a combination of atorvastatin and sildenafil generates an additive effect in promoting recovery after ischemic stroke in mice. We selected sildenafil instead of dipyridamole because sildenafil has been shown to have a neurorestorative effect, whereas there is no evidence of dipyridamole promoting neuroplasticity.

METHOD

Study Design

C57BL/6 adult mice were subjected to 60 minutes of transient middle cerebral artery occlusion (MCAO). The adhesive removal test was performed on postoperative day 1. In order to get a balanced baseline in each group, mice were randomized based on the adhesive removal test and stratified into four treatment groups: placebo (water), atorvastatin (0.3mg/kg), sildenafil (0.3mg/kg), or a combination of atorvastatin (0.3mg/kg) and sildenafil (0.3mg/kg). Drugs were administered by oral gavage starting 24 hours after ischemia and then daily for 6 days. The treatment was started 24 hours after ischemia to demonstrate the neurorestorative effect of the drugs¹⁴. Behavioral studies (neurological score and adhesive removal test) were performed at baseline (pre-surgery), on postoperative day 1, and on postoperative day 7. The cylinder test was performed at baseline and on postoperative day 7 because on postoperative day 1 animals were too weak to perform the test. To determine the histological tissue outcome, infarct volume and cerebral capillary length were measured.

Ischemia Model

Ischemic model—Adult male mice (wild-type C57 black/6 age 2–3 months, weight 22–25 g) were purchased from Charles River Laboratories (Wilmington, MA). All experiments

were performed following an institutionally approved protocol in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice were housed under diurnal lighting conditions and had free access to food and water. To generate focal cerebral ischemia, a transient monofilament occlusion method was used. Briefly, 2% isoflurane was used to induce anesthesia followed by 1% isoflurane with 70% N₂O/30% O₂ to maintain anesthesia. Rectal temperature was monitored and maintained at 37°C±0.5°C using a thermostatic heating pad (Frederick Haer and Co., Brunswick, ME). Blood flow at the middle cerebral artery territory was monitored using a flexible fiber optic probe (PF2B; Perimed, Stockholm, Sweden). To generate MCA occlusion, commercially available nylon filament (Doccol Corp) was inserted into the external carotid artery and advanced to the middle cerebral artery. The MCA occlusion was confirmed by >80% reduction of blood flow. The monofilament was removed after 60 minutes of occlusion.

Behavioral Study

Neurological score—General neurological deficit was assessed using a four-point (0–3) neurological score test: 0=no deficit, 1= failure to fully extend the forepaw, 2=circling to one side, 3=no spontaneous walking with a depressed level of consciousness.⁷

Adhesive removal test—The adhesive removal test measures sensorimotor function. A small piece of adhesive tape (3 mm × 4 mm) was placed on each forepaw, and the amount of time (seconds) needed to contact and remove the tape from each forepaw was recorded. Observation stopped at 4 minutes. Three runs were performed, and the best score was used to do analysis. Mice were trained three times prior to the surgery to ensure that they were able to remove the tape in fewer than 50 seconds.¹⁹

Cylinder test—The asymmetry of forelimb movements was measured using the cylinder test.²⁰ Mice were placed in a plexiglass cylinder. The number of contacts of left, right, or both forepaws with the cylinder wall was recorded. The recording was stopped after the total number of contacts reached 20 or after 15 minutes. The data point was excluded if animals had fewer than 10 contacts in 15 minutes. The test was performed at baseline prior to surgery and on post-operative day 7. The lateralization index was calculated as (number of right side placement - number of left side placement)/total placement.

Infarct Size Measurement

To measure the infarct size, fresh-frozen brain sections were stained using hematoxylin-eosin (H&E). The infarct volume was measured with an imaging system (MCID, Interfocus Imaging, Cambridge, UK). Indirect volumes in percent of the contralateral side were calculated.³

CD 31 Immunohistochemistry

Cerebral vascular endothelium cells were labeled with an antibody against CD31 (n= 7–8 mice/group) as described.²¹ Brains were fresh-frozen and sectioned with a cryostat. Sections were stained with mouse anti-CD31 monoclonal antibody overnight (1:200 dilution, Abcam, Cambridge, MA). A secondary antibody that was used was anti-mouse goat Cy3 conjugates (1:500 dilution, Jackson ImmunoResearch laboratories, West Grove, PA). To measure the length of CD-31 positive blood vessels, four sections from each brain at Bregma 0.9, 0.6, 0.3, and –0.1 were obtained. Three areas in each brain section were measured: striatum in contralateral, infarct, and peri-infarct. Four fields were selected at approximately the same location in each area, and the total length of CD-31 positive vessels (mm) within the field was measured using image analysis software (MCID Elite). The measurement was conducted by an investigator that was blinded to the treatment.

Statistical Analysis

Mortality rate was analyzed using Chi square analysis. Data were reported as mean $\pm$ SD, except for neurological scores that were reported as median (range). Based on our hypothesis that combination treatment is better than individual drug-treatment group in outcome variables including adhesive removal test and cylinder test, we used linear regression model²² to compare the combination treatment group with the individual-drug treatment group and placebo on postoperative day 7 for adhesive removal test and cylinder test. For adhesive removal test, we used linear regression with censoring (Tobit regression model) because the outcome variables are censored (maximum score is 240 seconds). Two-way ANOVA was performed to analyze the difference in body weight and neurological scores on postoperation day 1 and postoperation day 7. ANOVA was performed to analyze the difference in infarct volume, cerebral capillary length among all four groups because the null hypothesis is that there is no difference among the groups in the above outcomes. P values <0.05 were considered significant. There was no correction for multiple testing.

RESULTS

Mortality

A total of 67 mice were included in the study. Mortality rate ranged from 35% to 41% in each group and there was no significant difference among the groups ($p=0.983$, Table 1 and Figure 1). Mice that died within 24 hours after MCAO were excluded from the study. Mice that were included but died before the 7th day were excluded from behavioral and tissue outcome studies.

Body Weight

Body weight was measured after the MCAO. The percentage of body weight to pre-surgery weight is reported in Table 2. All groups showed weight reduction during the course of 7 days after MCAO. Two way ANOVA showed no significant difference for either the time factor $F(1, 17)=15$, $p>0.05$ or the treatment factor $F(3,17)=0.082$, $p>0.05$.

Functional Outcome

To measure functional outcome, neurological score, adhesive removal test, and cylinder test were performed. For neurological score, animals displayed improved function from postoperative day 1 to postoperative day 7 regardless of treatment (time factor $F(1, 38)=240$, $p<0.01$, two-way ANOVA). There were no significant differences among the treatment groups (treatment factor $F(3, 38)=0.49$, $p>0.05$, Table 3). For adhesive removal test, Tobit regression revealed that on postoperative day 7, the combination group was significantly better in the removal time (seconds) of adhesive tape on the lesioned limb (50 ± 54) as compared to the control group (147 ± 109) ($P<0.05$) and to atorvastatin (144 ± 102) ($p<0.05$), but did not show statistically significant improvement compared to sildenafil (107 ± 115) ($p=0.148$) (Table 4). On the other hand, neither atorvastatin nor sildenafil alone improved the adhesive removal test as compared to control ($P>0.05$). There was no significant difference in the contact time in each limb or the removal time in the normal limb (as seen in supplementary data Table 1–3). For the cylinder test, the lateralization index was 0.3 ± 0.35 in the vehicle group, -0.057 ± 0.36 after atorvastatin treatment, -0.046 ± 0.36 after sildenafil treatment, and 0.07 ± 0.24 for combination treatment. There was no significant difference for combination compared to placebo or individual treatment using linear regression analysis (Table 5). For the sildenafil treatment group, one data point was excluded because the animal touched the cylinder walls only 5 times in 15 minutes.

Infarct Volume

Indirect infarct volume was measured as described³. As shown in Table 6, there was no significant difference among the groups as analyzed using ANOVA.

Capillary Length

As shown in Table 7 and Figures 2A–2C, there was no difference in total capillary lengths among the 4 groups as analyzed using ANOVA.

DISCUSSION

Our data suggest that a combination of atorvastatin and sildenafil at subclinical dose is significantly better than atorvastatin alone on a single variable (adhesive removal test) out of multiple variables tested. The adhesive removal study tests sensory and fine motor function. The latter involves both forepaws and mouth. The removal time from the hemiplegic limb was significantly improved in the combination treatment group; whereas there was no significant difference in contact time. The data suggest the improvement might be due to improvement in fine motor function but not sensory function. Gross motor function as measured by neurological score and cylinder test did not show any difference among the groups.

However, combination treatment was not better than sildenafil alone. It is possible that the effect of combination treatment was mostly due to the effect of sildenafil. However, at this subclinical dose, neither atorvastatin nor sildenafil alone improved sensorimotor function as compared to control, which suggests that there is an additive effect of the two drugs. The enhanced efficacy of the combination of atorvastatin and sildenafil has been demonstrated in other systems, including myocardial infarction^{16, 17}, erectile dysfunction²³, and hypertension.²⁴ The data suggest that combination treatment might be potentially a more potent therapy than individual-drug treatment.

Because mice have good spontaneous recovery after stroke and motor function tests may show a ceiling effect, we used subclinical doses of atorvastatin and sildenafil in order to detect the difference between combination treatment and individual-drug treatment.

The mechanism of this combination in promoting stroke recovery is not clear. It is possible that a combination of atorvastatin and sildenafil induces a synergistic effect by activating the NO/cGMP pathway. Atorvastatin induces NO production, which leads to the generation of cGMP. Sildenafil inhibits cGMP hydrolysis, which leads to the accumulation of cGMP.²⁵ NO causes vessel dilation, which leads to improved cerebral perfusion⁷, and induces angiogenesis²⁶ as well as neurogenesis.²⁷ In addition, NO also plays a role in reducing brain vascular inflammation and leakage.²⁸ All of the above functions have been shown to contribute to brain plasticity and functional recovery after stroke. Improved cerebral perfusion⁷, blood brain barrier repair²⁹, and reduced inflammation³⁰ have been shown to play important roles in early phase stroke recovery. Angiogenesis and neurogenesis are likely to play a role in late phase recovery. Our data show improved motor recovery at 7 days after ischemia without affecting capillary length. This may indicate that early events such as cerebral perfusion, blood brain barrier repair, and inflammation reduction might play major roles in this recovery. Furthermore, as expected, the treatment did not affect infarct volume, probably because the treatment was started 24 hours after ischemia.

In conclusion, the data suggest that combined atorvastatin and sildenafil might be a better treatment regimen than single-drug treatment. However, the effects observed are not robust. Further studies are needed to optimize the treatment conditions, such as dosing, timing, and duration of the combination treatment and to elucidate the mechanism of action.

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Figure 1. Cumulative daily mortality rate. Cumulative daily mortality rate=cumulative daily mortality/total mice subjected to MCAO in each group.

Figure 2.

A–C: CD31 staining. To measure capillary length, brain sections were immunoblotted with mouse anti-CD31 antibody followed by anti-mouse Cy3 conjugated secondary antibody. The length of CD-31 positive blood vessels was measured using an image analysis software.

Table 1

Mortality

Treatment Group	Died	Total	Mortality Rate (%)
Placebo	6	17	35
Sildenafil	7	17	41
Atorvastatin	6	16	38
Sildenafil + atorvastatin	6	17	35

Pearson's χ^2 = 0.168; P = 0.983.

Table 2

Body weight

Groups	POD1, % (Mean $\pm$ SD)	POD7, % (Mean $\pm$ SD)
Placebo	88 $\pm$ 0.04	82 $\pm$ 0.2
Sildenafil	85 $\pm$ 0.02	76 $\pm$ 0.1
Atorvastatin	89 $\pm$ 0.02	71 $\pm$ 0.1
Sildenafil + atorvastatin	88 $\pm$ 0.02	79 $\pm$ 0.1

Body weight on postoperative day (POD) 1 and POD7 is expressed as percentage of presurgery weight. Two-way ANOVA showed no significant difference for either time factor $F(1, 17) = 15$, ($P > 0.05$) or treatment factor $F(3, 17) = 0.82$ ($P > 0.05$).

Table 3

Neurologic score

Groups	POD1 Median (Range)	POD7 Median (Range)
Placebo	2 (1–3)	0 (0–1)
Sildenafil	2 (1–3)	0 (0–2)
Atorvastatin	2 (1–2)	0 (0–1)
Sildenafil + atorvastatin	2 (1–2)	0 (0–1)

Body weight on postoperative day (POD) 1 and POD7 is expressed as percentage of presurgery weight. Two-way ANOVA showed no significant difference for either the time factor $F(1, 17) = 15$ ($P > 0.05$) or treatment factor $F(3, 17) = 0.82$ ($P > 0.05$).

Table 4

Adhesive removal test

	Baseline	POD 1	POD 7	P Value (Combination v Placebo, Sildenafil, Atorvastatin) on POD 7
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Placebo (n = 11)	11 $\pm$ 6	174 $\pm$ 109	147 $\pm$ 109	0.017 (combination v placebo)
Sildenafil (n = 10)	16 $\pm$ 8	177 $\pm$ 95	107 $\pm$ 115	0.148 (combination v sildenafil)
Atorvastatin (n = 9)	12 $\pm$ 6	173 $\pm$ 101	144 $\pm$ 102	0.047 (combination v atorvastatin)
Atorvastatin + sildenafil (n = 11)	18 $\pm$ 5	181 $\pm$ 85	50 $\pm$ 54	

A small piece of adhesive tape was placed on the hairless part of the forepaw bilaterally. The time in seconds that the animals took to remove the adhesive tape on hemiplegic forepaw is reported. The maximum time recorded was 240 secs. Linear regression with censoring was used to compare combination with placebo, sildenafil, and atorvastatin groups on postoperative day (POD) 7.

Table 5

Cylinder test

	POD7	P Value (Combination v Placebo, Sildenafil, Atorvastatin) on POD 7
	Mean $\pm$ SD	
Placebo (n = 11)	0.3 $\pm$ 0.35	0.087 (combination v placebo)
Sildenafil (n = 9)	-0.046 $\pm$ 0.42	0.458 (combination v sildenafil)
Atorvastatin (n = 10)	-0.057 $\pm$ 0.36	0.403 (combination v atorvastatin)
Atorvastatin + sildenafil (n = 11)	0.07 $\pm$ 0.24	

Lateralization index was calculated as (number of right side placement -- number of left side placement)/total placement. Mean for each group was used for statistical analysis. Combination was compared with placebo or individual treatment using linear regression. POD indicates postoperative day.

Table 6

Infarct volume

	Indirect/Contralateral, %
	Average $\pm$ SD
Placebo (n = 11)	21 $\pm$ 8
Sildenafil (n = 10)	25 $\pm$ 10
Atorvastatin (n = 10)	22 $\pm$ 8
Atorvastatin + sildenafil (n = 11)	25 $\pm$ 11
ANOVA test	P = 0.75

On postoperative day (POD) 7, brain slices were stained with hematoxylin and eosin, and percentage of indirect infarct volume of contralateral was calculated. ANOVA was used for statistical analysis.

Table 7

Quantitation of cerebral capillary length

	Contralateral Striatum, mm	Infarct Striatum, mm	Periinfarct Striatum, mm
Placebo (n = 7)	9108 ± 1654	5008 ± 3218	6592 ± 2445
Sildenafil (n = 7)	9965 ± 935	5880 ± 1924	6959 ± 1588
Atorvastatin (n = 7)	9178 ± 2365	5716 ± 2516	6971 ± 2393
Sildenafil + atorvastatin (n = 8)	8631 ± 2378	5930 ± 2421	6778 ± 2508
ANOVA test	P = 0.64	P = 0.89	P = 0.99

Cerebral vascular was stained with anti-CD 31 antibody on fresh-frozen brain section. The measurement was performed using MCID software. The sum of the length measured in each brain is presented. One-way ANOVA was used to compare all 4 groups for each region.

Appendix Table I

Right side tape contact time in the adhesive removal test

	POD 1	POD 7	P Value (Combination v Placebo, Sildenafil, Atorvastatin) on POD 7
	Mean $\pm$ SD	Mean $\pm$ SD	
Placebo (n = 11)	7 $\pm$ 7	18 $\pm$ 35.9	0.21 (combination v placebo)
Sildenafil (n = 10)	6 $\pm$ 7	4 $\pm$ 3	0.35 (combination v sildenafil)
Atorvastatin (n = 9)	76 $\pm$ 104	27 $\pm$ 75	0.33(combination v atorvastatin)
Atorvastatin + sildenafil (n = 11)	8 $\pm$ 5	5 $\pm$ 3	

A small piece of adhesive tape was placed on the hairless part of each forepaw. The time in seconds that the animals took to contact and remove the adhesive tape on the forepaw was recorded. The maximum time recorded was 240 seconds. Linear regression with censoring (Tobit regression) showed no significant difference as combination treatment compared with placebo, sildenafil, or atorvastatin groups on postoperative day (POD) 7.

Appendix Table II

Left side tape contact time in the adhesive removal test

	POD 1	POD 7	P Value (Combination v Placebo, Sildenafil, Atorvastatin) on POD 7
	Mean $\pm$ SD	Mean $\pm$ SD	
Placebo (n = 11)	69 $\pm$ 89	64 $\pm$ 93	0.05 (combination v placebo)
Sildenafil (n = 10)	10 $\pm$ 8	27 $\pm$ 75	0.15 (combination v sildenafil)
Atorvastatin (n = 9)	73 $\pm$ 96	20 $\pm$ 29	0.39 (combination v atorvastatin)
Atorvastatin + sildenafil (n = 11)	33 $\pm$ 68	7 $\pm$ 5	

Tobit regression showed no significant difference as combination treatment compared with placebo, sildenafil, or atorvastatin groups on postoperative day (POD) 7.

Appendix Table III

Right side tape removal time in the adhesive removal test

	POD 1	POD 7	P Value (Combination v Placebo, Sildenafil, Atorvastatin) on POD 7
	Mean $\pm$ SD	Mean $\pm$ SD	
Placebo (n = 11)	130 $\pm$ 98	37 $\pm$ 68	0.78 (combination v placebo)
Sildenafil (n = 10)	113 $\pm$ 118	36 $\pm$ 72	0.83 (combination v sildenafil)
Atorvastatin (n = 9)	168 $\pm$ 108	44 $\pm$ 62	0.62 (combination v atorvastatin)
Atorvastatin + sildenafil (n = 11)	108 $\pm$ 109	31 $\pm$ 50	

Tobit regression showed no significant difference as combination treatment compared with placebo, sildenafil, or atorvastatin groups on postoperative day (POD) 7.