

Combination Therapy with Fenofibrate, a Peroxisome Proliferator-Activated Receptor α Agonist, and Simvastatin, a 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Inhibitor, on Experimental Traumatic Brain Injury

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ABSTRACT

We and others have demonstrated that fibrates [peroxisome proliferator-activated receptor (PPAR) α agonists] and statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) exerted neuroprotective and pleiotropic effects in experimental models of traumatic brain injury (TBI). Because the combination of statins and fibrates synergistically enhanced PPAR α activation, we hypothesized that the combination of both drugs may exert more important and/or prolonged beneficial effects in TBI than each alone. In this study, we examined the combination of fenofibrate with simvastatin, administered 1 and 6 h after injury, on the consequences of TBI. First, our dose-effect study demonstrated that the most efficient dose of simvastatin (37.5 mg/kg) reduced post-traumatic neurological deficits and brain edema. Then, the effects of the combination of fenofibrate (50

mg/kg) and simvastatin (37.5 mg/kg), given p.o. at 1 and 6 h after TBI, were evaluated on the TBI consequences in the early and late phase after injury. The combination exerted more sustained neurological recovery-promoting and antiedematous effects than monotherapies, and it synergistically decreased the post-traumatic brain lesion. Furthermore, a delayed treatment given p.o. at 3 and 8 h after TBI with the combination was still efficient on neurological deficits induced by TBI, but it failed to reduce the brain edema at 48 h. The present data represent the first demonstration that the combination of fenofibrate and simvastatin exerts prolonged and synergistic neuroprotective effects than each drug alone. Thus, these results may have important therapeutic significance for the treatment of TBI.

Traumatic brain injury (TBI) remains one of the leading causes of death and disability in industrialized countries. Despite numerous studies on animal models of TBI that investigated therapeutic strategies, no neuroprotective therapy is currently available (Bramlett and Dietrich, 2004). TBI leads to important and deleterious neuroinflammation, as evidenced by edema, free radicals, cytokine production, induction of nitric-oxide synthase and cyclooxygenase type 2, and leukocyte infiltration. Strategies blocking each inflammatory and oxidative mediator have been shown to induce neuroprotective, anti-inflammatory and antioxidative effects after brain injury (Ray et al., 2002). Drugs that were able to modulate only one molecular pathway implicated in inflam-

mation or oxidative stress induced beneficial effects in experimental studies, but they failed in clinical trials. Thus, one key to success in neuroprotection would be to simultaneously modulate many pathophysiological pathways with pharmacological agent inducing pleiotropic effects.

One pleiotropic strategy is to activate peroxisome proliferator-activated receptor (PPAR) α . PPAR α is one of the three subtypes of the nuclear receptor PPAR family, which can be activated by natural ligands, such as polyunsaturated fatty acids, and synthetic ligands (drugs belonging to fibrate's family). PPARs are implicated in several physiological processes, such as regulation of lipoprotein, lipid metabolism, and glucose homeostasis. In addition to their broad use as lipid-lowering drugs, PPAR α activators induce pleiotropic effects on inflammation and oxidative stress (Fruchart et al., 1999). Indeed, we recently demonstrated that 50 mg/kg fenofibrate, a synthetic PPAR α agonist widely used as a lipid-

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ABBREVIATIONS: TBI, traumatic brain injury; PPAR, peroxisome proliferator-activated receptor; NF- κ B, nuclear factor- κ B; BWC, brain water content; ANOVA, analysis of variance.

lowering drug, induced neurological recovery-promoting, antiedematous, and neuroprotective effects after TBI associated with anti-inflammatory and antioxidant effects (Besson et al., 2005; Chen et al., 2007b).

Statins, another lipid-lowering drugs, inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase and, thereby, suppress cholesterol biosynthesis. Apart from their lipid-lowering activities, statins have also been shown *in vitro* and *in vivo* to mediate pleiotropic effects by reducing inflammation and oxidative stress (Liao and Laufs, 2005; Greenwood and Mason, 2007). Several studies showed that statins induced neurological recovery and neuroprotective and anti-inflammatory effects after experimental TBI (Qu et al., 2005; Chen et al., 2007a; Lu et al., 2007; Wang et al., 2007). *In vitro* statins increased the expression and activity of PPAR α (Inoue et al., 2000; Martin et al., 2001; Landrier et al., 2004; Zapolska-Downar et al., 2004). *In vivo*, in two acute inflammation models, Paumelle et al. (2006) showed that simvastatin (a widely used statin) exerted anti-inflammatory effects through activation of PPAR α . These data demonstrated a cross-talk of statin-signaling pathways and PPAR α . More interestingly, an *in vitro* combination of statins and fibrates synergistically enhanced PPAR α activity (Martin et al., 2001) and inhibited the transcriptional activation of nuclear factor κ B (NF- κ B) (Inoue et al., 2002). In patients with hypercholesterolemia, both simvastatin and fenofibrate, when given alone demonstrated an anti-inflammatory effect, as evidenced by a decrease in plasma levels of tumor necrosis factor α , matrix metalloproteinase 9, and C-reactive protein (Staels et al., 1998; Musial et al., 2001; Koh et al., 2004). Moreover, in these patients, combined therapy with statins and fibrates was more effective at controlling atherogenic dyslipidemia than either drug alone, suggesting a better efficiency of the combined therapy (Athysos et al., 2002; Vega et al., 2003; Koh et al., 2005).

Because statins and fibrates, when given alone, promoted neuroprotective effects after TBI, and, to date, there is no information about their combination, we hypothesized that such combination may exert more important beneficial effects in TBI than each alone. To test this hypothesis, we studied the effects of the combination of fenofibrate with simvastatin in the lateral fluid-percussion TBI model.

In the literature, only long-term treatment with statins has been investigated in TBI (Chen et al., 2007a; Lu et al., 2007). Therefore, in the first part of this study, to investigate the effect of an acute treatment of statin in TBI, we studied the dose effect of simvastatin on neurological deficits and brain edema induced by TBI. Then, in the second part of this work, we examined whether the combination of fenofibrate and simvastatin on TBI is more efficient at reducing TBI-induced consequences than each drug alone.

Materials and Methods

Animals and Materials

Animal care and experimentation (authorization no. 75-776) complied with both French and European Community regulations (Official Journal of European Community L358 12/18/1986) and conformed to the Institute of Laboratory Animal Resources (1996). Male Sprague-Dawley rats were supplied by Elevage Janvier Laboratories (Le Genest-St.-Isle, France). Fenofibrate, simvastatin, and methylcellulose were purchased from Sigma Chemicals Corporation (Saint Quentin-Fallavier, France).

Fluid Percussion-Induced Brain Injury

Male Sprague-Dawley rats (weighing 300–360 g) were anesthetized with chloral hydrate (400 mg/kg *i.p.*) and placed on a stereotaxic frame. During surgery, the animals were positioned on a heating blanket (Harvard Apparatus, Les Ulis, France) to maintain body normothermia ($37.5 \pm 0.5^\circ\text{C}$). TBI of moderate severity was induced by fluid percussion using the protocol described previously (Besson et al., 2003). The scalp was incised, and a 3-mm craniotomy was made lateral to the right temporoparietal cortex (coordinates: 3.5 mm anterior and 6 mm above the interaural line) with a dental drill, taking care to leave the dura mater intact. A 3-mm diameter polyethylene tube was placed over the dura mater, fixed securely into the craniotomy site with dental cement (Meliodent; Bayer, Berkshire, UK), and connected to a solenoid valve (Danfoss, Nordborg, Denmark). The opposite end of the valve was connected to a high-performance liquid chromatography pump (Gilson, Villier Le Bel, France). The system was filled with sterile water, providing a calibrated outflow pressure of 1.6 to 1.8 bar. A 20-ms solenoid valve opening controlled with a timer (Omron, Tokyo, Japan) triggered the percussion directly onto the dura mater. The applied cortical pressure was measured extracranially by a pressure transducer (emka Technologies, Paris, France) connected to an oscilloscope (DSO 400; Gould, Les Ulis, France). Immediately after fluid percussion, the tube was removed, the scalp was sutured, and the animal was returned to its home cage (warmed at $26\text{--}28^\circ\text{C}$) to recover from the anesthesia. Thereafter, rats were group-housed under temperature- and light-controlled conditions with access to food and water *ad libitum*. Sham-operated rats underwent the same surgery except for percussion.

Experimental Protocols

Experiment 1: Dose-Effect Study of Simvastatin on Neurological Deficits and Brain Edema Induced by TBI. Paumelle et al. (2006) reported that an acute dose regimen of simvastatin at 50 mg/kg exerted antiedematous and anti-inflammatory effects in two different acute inflammatory models. Therefore, we chose the dose of 50 mg/kg and framed it with inferior and superior doses. Rats were given simvastatin (25, 37.5, 50, 75, and 100 mg/kg) and its vehicle *p.o.* at 1 and 6 h post-TBI. Sham-operated animals received vehicle *p.o.* with the same schedule. Because we previously showed that fenofibrate (administered at 50 mg/kg at 1 and 6 h after TBI) exerted neuroprotective, anti-inflammatory and antioxidants effects (Besson et al., 2005; Chen et al., 2007b), we designed the same schedule of treatment for simvastatin, keeping in mind its future use as combined therapy with fenofibrate. Moreover, simvastatin has a hydrophobic property, a high blood-brain barrier penetration, and a half-life of 4 h. Thus, all of these data led us to choose this schedule of treatment.

The neurological assessments were done at 6 and 24 h after TBI, and then rats were killed for brain edema measurement.

Experiment 2: Effects of Combination on Neurological Deficits, Brain Edema, and Lesion Induced by TBI. Because we previously showed that fenofibrate at 50 mg/kg (administered at 1 and 6 h after TBI) exerted neurological recovery and antiedematous, neuroprotective, anti-inflammatory, and antioxidant effects (Besson et al., 2005; Chen et al., 2007b), we chose this dose and this schedule of treatment for the combination therapy study.

Moreover, recent studies showed that combined oral administration of fenofibrate and simvastatin is devoid of any possible pharmaceutical or intestinal absorption interactions after single oral doses (Penn et al., 2006).

To evaluate the acute effects of combination on neurological deficits and brain edema induced by TBI, rats were given either vehicle, 50 mg/kg fenofibrate, 37.5 mg/kg simvastatin, or the combination *p.o.* at 1 and 6 h after injury. Sham-operated animals received vehicle *p.o.* with the same schedule. The neurological assessments were done at 6, 24, and 48 h after TBI. Brain edema was determined at 24 and 48 h post-TBI.

The same schedule of treatment was used to evaluate the long-term effects of combination on neurological deficits and brain lesion induced by TBI. The neurological assessments were performed 3 and 7 days after TBI, and then rats were killed for brain lesion determination.

To evaluate the effects of a delayed treatment with monotherapies and combination on neurological deficits and brain edema induced by TBI, rats were given vehicle, 50 mg/kg fenofibrate, 37.5 mg/kg simvastatin, or the combination p.o. at 3 and 8 h after TBI. Neurological assessments were performed at 8, 24, and 48 h after injury, then rats were killed for brain edema measurement.

Fenofibrate, simvastatin, and the combination were suspended in water containing 0.5% methylcellulose (vehicle).

Neurological Score

A neurological examination was performed in a blinded fashion using a grading scale (Besson et al., 2003). Contralateral sensorimotor functions were examined by assessing placing reactions (leg hanging and visual), grasping reflex, and righting reflex in rats placed on a table. Rats were also examined for abnormal postures (forelimb flexion and thorax twisting). The scores for each item were summed and used as a global neurological score; the maximum was 9 for nonoperated rats.

Beam-Walking Score

This score evaluates motor coordination of animals. The test was performed as described by Millerot-Serruot et al. (2007). The wooden beam was 70-cm long and 2.5-cm wide and was placed 100 cm above the floor. One end of the beam was connected to a black box (28 × 25 × 25 cm) with an opening to encourage the rat to move along the beam. The beam-walking performance was scored in a blinded fashion using a grading scale from 0 to 4 as follows: 0, the rat falls down or is unable to traverse the beam even after a stimulus (noise or slap in the hindquarters); 1, the rat can traverse the beam, but the affected forelimb and/or hindlimb does not aid in locomotion; 2, the rat traverses the beam with more than three footslips (any use of the forelimb or hindlimb on the side of the beam, or if either foot slipped off the top surface of the beam) or with an abnormal posture; 3, the rat crosses the beam normally but only after a stimulus (noise or slap in the hindquarters); and 4, the rat crosses the beam normally without exogenous stimulus. All rats were trained to traverse the beam 24 h before and on the day of injury immediately before anesthesia. The maximum was 4 for nonoperated rats.

Brain Edema

Cerebral edema was determined by measuring brain water content (BWC) using the wet weight – dry weight technique, and the results are expressed as a percentage of the water content. Rats were killed by an overdose of sodium pentobarbital (200 mg/kg i.p.). The brains were promptly removed, and a thick (4 mm) coronal slice was taken at the temporoparietal level. The slice was removed from the ipsilateral hemisphere. The fresh tissue samples were immediately weighed (wet weight) and placed in an incubator at 100°C for 24 h. The samples were weighed once again to determine the dry weight. The BWC was calculated as follows:

$$\text{BWC} = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100\%$$

Brain Lesion

At 7 days post-TBI, rats were anesthetized with pentobarbital and killed by decapitation. Their brains were promptly removed, frozen in isopentane, and stored at –40°C. Serial coronal sections (50- μm thick) were cut in a cryostat (–15°C) at 1-mm intervals, beginning at the level 13.7 to 1.7 mm relative to the interaural line. The sections were stained with cresyl violet. The lesion areas (in mm^2) were measured with an image analyzer (IMSTAR, Paris, France), and the distances between respective coronal sections were used to calculate

the lesion volume. The brain lesion volume (in mm^3) was calculated by integrating the necrotic areas, corrected for edema, by the method of Golanov and Reis (1995).

Statistical Analysis

Data are presented as the mean \pm S.E.M. Differences in neurological and beam-walking scores were determined by nonparametric Kruskal-Wallis analysis followed by a Mann-Whitney *U* test. Differences in temperature, BWC, and brain lesion volume were evaluated by one-way analysis of variance (ANOVA) with subsequent group comparisons by Fisher's protected least significant difference (PLSD) test. A *p* value of 0.05 was considered to be the threshold for a significant difference.

Results

Dose-Effect of Simvastatin on Neurological Function, Motor Coordination, and Brain Edema after TBI.

Nonoperated rats had a neurological score of 9 (Fig. 1A). Those of sham-operated rats were not different at 6 and 24 h. TBI led to a decrease in the neurological score at 6 h after injury (4.9 ± 0.3 , $p < 0.001$), demonstrating a neurological deficit that persisted at 24 h (4.2 ± 0.3 , $p < 0.001$). At 6 h after injury, rats that were given 50 and 100 mg/kg simvastatin had an increase in the neurological score (50 mg/kg: 6.4 ± 0.5 , $p < 0.05$; 100 mg/kg: 7.2 ± 0.5 , $p < 0.01$), showing a neurological recovery. Other doses did not modify the neurological score compared with the vehicle-treated injured rats. At 24 h after injury, rats that were given 37.5 mg/kg simvastatin showed an improvement of the neurological score (6.6 ± 1.1 , $p < 0.05$). Others doses did not modify the neurological score.

Nonoperated rats had a beam-walking score of 4 (Fig. 1B). There was no difference between nonoperated and sham-operated rats at 6 and 24 h. TBI led to a significant decrease in the beam-walking score at 6 h after injury (1.0 ± 0.3 , $p < 0.001$), demonstrating a post-traumatic deficit of motor coordination that persisted at 24 h (0.6 ± 0.2 , $p < 0.01$). At 6 h postinjury, the simvastatin at the studied doses had no effect on the beam-walking score. At 24 h post-TBI, rats that were treated with 37.5 mg/kg simvastatin improved the beam-walking score (2.8 ± 0.8 , $p < 0.05$).

The BWC of nonoperated rats was $79.4 \pm 0.1\%$ (Fig. 2). Those of sham-operated rats were not different at 24 h. TBI led to an increase of the BWC at 24 h after injury ($82.6 \pm 0.4\%$, $p < 0.001$), demonstrating brain edema. At 24 h post-TBI, the post-traumatic BWC was reduced by simvastatin at 37.5 mg/kg ($80.6 \pm 0.5\%$, $p < 0.01$), 50 mg/kg ($81.3 \pm 0.4\%$, $p < 0.05$), and 75 mg/kg ($81.1 \pm 0.5\%$, $p < 0.05$).

These first data enabled us to determine that the dose of 37.5 mg/kg simvastatin is the most efficient one because it reduced the post-traumatic neurological and motor coordination dysfunctions and brain edema. Thus, we chose it for the combined therapy studies.

Effects of Fenofibrate, Simvastatin, and the Combination on Rectal Temperature. Temperature of the animals was measured during surgery and recovery. There was no difference between the vehicle- and drug-treated groups during surgery, at 1, 6, 24, and 48 h after TBI (Table 1).

Effects Of the Combination on Neurological Function and Motor Coordination at 6, 24, and 48 h after TBI. Nonoperated rats had a neurological score of 9 (Fig. 3A). There was no difference between nonoperated and sham-

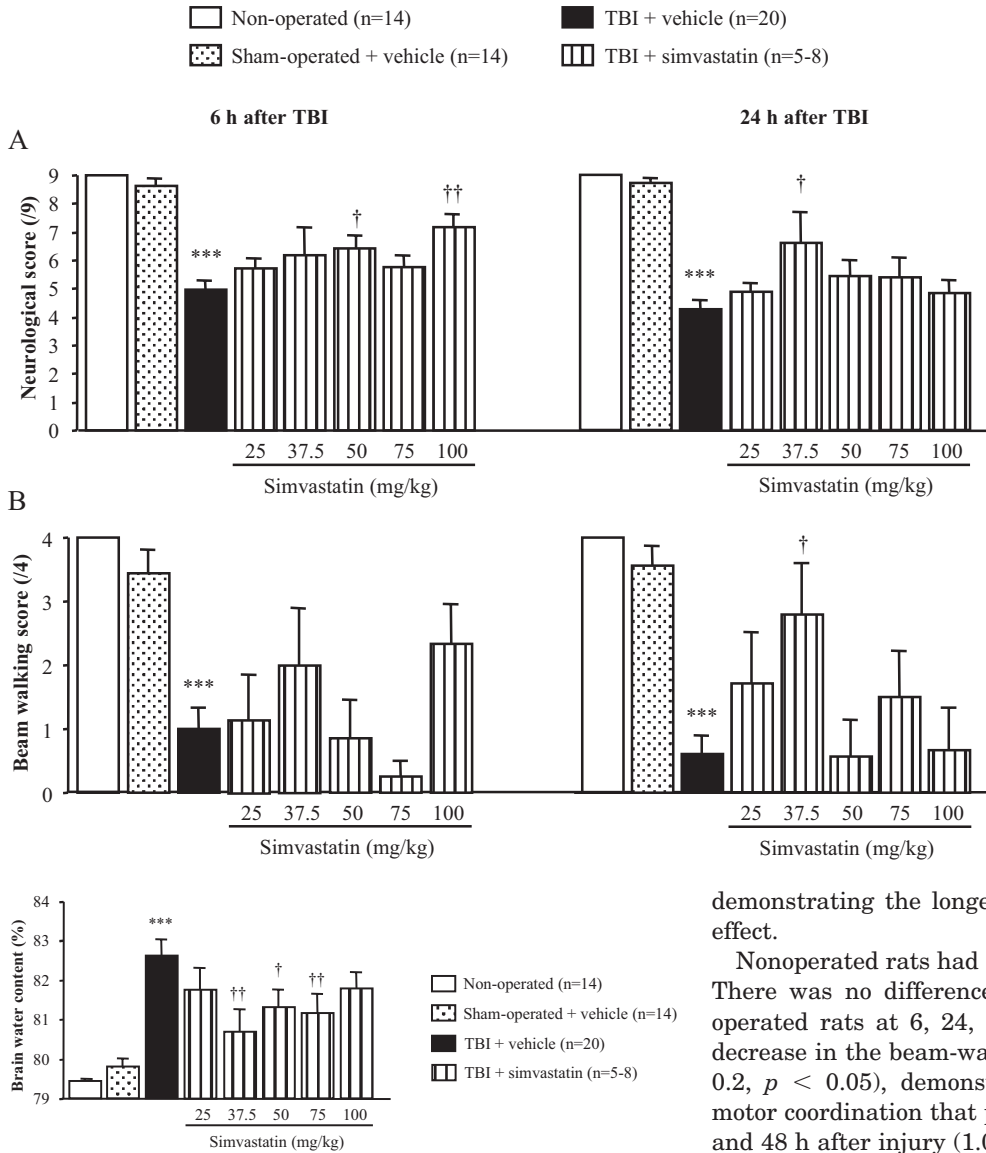


Fig. 1. Effects of simvastatin on the neurological (A) and beam-walking (B) scores at 6 and 24 h after TBI. Simvastatin (25, 37.5, 50, 75, and 100 mg/kg) or its vehicle (water containing 0.5% methylcellulose) were administered p.o. at 1 and 6 h after TBI. A, TBI led to a decrease in the neurological score at 6 h after injury that persisted at 24 h. At 6 h after injury, rats that were given 50 mg/kg and 100 mg/kg simvastatin had an increase in the neurological score. At 24 h after injury, rats that were given 37.5 mg/kg simvastatin showed an improvement of the neurological score. B, TBI led to a decrease in the beam-walking score at 6 h postinjury that persisted at 24 h. Rats treated with 37.5 mg/kg simvastatin showed an improved beam-walking score at 24 h after injury. Data are presented as the mean ± S.E.M. Differences in neurological and beam-walking scores were determined by nonparametric Kruskal-Wallis analysis followed by a Mann-Whitney *U* test. ***, *p* < 0.001 versus sham-operated; †, *p* < 0.05 and ††, *p* < 0.01 versus TBI + vehicle.

Fig. 2. Effects of simvastatin on the brain water content 24 h after TBI. Simvastatin (25, 37.5, 50, 75, and 100 mg/kg) or its vehicle (water containing 0.5% methylcellulose) were administered p.o. at 1 and 6 h after TBI. TBI led to an increase of the brain water content 24 h after injury, which was reduced by simvastatin at 37.5, 50, and 75 mg/kg. Data are presented as the mean ± S.E.M. Differences in brain water content were evaluated by one-way ANOVA with subsequent group comparisons by PLSD Fisher's test. ***, *p* < 0.001 versus sham-operated; †, *p* < 0.05 and ††, *p* < 0.01 versus TBI + vehicle.

operated rats at 6, 24, and 48 h. TBI led to a decrease in the neurological score at 6 h after injury (4.4 ± 0.4 , *p* < 0.001), demonstrating a neurological deficit that persisted at 24 (3.9 ± 0.4 , *p* < 0.001) and 48 h after injury (3.9 ± 0.4 , *p* < 0.001). Rats that were treated with fenofibrate (6.5 ± 0.7 , *p* < 0.05), simvastatin (6.5 ± 0.4 , *p* < 0.01), or their combination (5.9 ± 0.4 , *p* < 0.05) showed an increase in the neurological scores at 6 h post-TBI that persisted at 24 h (fenofibrate: 5.5 ± 0.7 , *p* < 0.05; simvastatin: 6.7 ± 0.7 , *p* < 0.01; combination: 6.3 ± 0.3 , *p* < 0.01), showing a neurological recovery. Even if monotherapies had no more effects on the neurological score at 48 h post-TBI, combination still showed an improvement of the neurological score (6.9 ± 0.4 , *p* < 0.001),

demonstrating the longest neurological recovery-promoting effect.

Nonoperated rats had a beam-walking score of 4 (Fig. 3B). There was no difference between nonoperated and sham-operated rats at 6, 24, and 48 h. TBI led to a significant decrease in the beam-walking score at 6 h postinjury (0.8 ± 0.2 , *p* < 0.05), demonstrating a post-traumatic deficit of motor coordination that persisted at 24 (0.8 ± 0.3 , *p* < 0.01) and 48 h after injury (1.0 ± 0.4 , *p* < 0.01). Both monotherapies and combination had no effects on the beam-walking scores at 6 and 24 h post-TBI. At 48 h postinjury, rats receiving the combination showed an improvement of the beam-walking score (2.7 ± 0.5 , *p* < 0.05), whereas monotherapies had no effects.

Effects of the Combination on Cerebral Edema at 24 and 48 h after TBI. The BWC of nonoperated rats was $79.6 \pm 0.1\%$ at 24 h (Fig. 4A) and $79.7 \pm 0.1\%$ at 48 h (Fig. 4B). Those of sham-operated rats were not different at 24 and 48 h. TBI led to an increase of the BWC 24 h after injury ($83.9 \pm 0.8\%$, *p* < 0.001), demonstrating brain edema that persisted at 48 h after TBI ($83.2 \pm 0.5\%$, *p* < 0.001). At 24 h post-TBI, the post-traumatic BWC was reduced by fenofibrate ($81.7 \pm 0.7\%$, *p* < 0.05), simvastatin ($81.8 \pm 0.5\%$, *p* < 0.05), and their combination ($81.5 \pm 0.6\%$, *p* < 0.01). There were no differences in BWC decrease between monotherapies and combination. At 48 h post-TBI, the combination reduced the BWC ($81.5 \pm 0.5\%$, *p* < 0.05), whereas both monotherapies did not reduce it any more.

Effects of the Combination on Neurological Function and Motor Coordination 3 and 7 Days after TBI. TBI induced a decrease in the neurological score (3.8 ± 0.5 ,

TABLE 1
Rectal temperature of the animals during surgery, and 1, 6, 24, and 48 h after surgery

| Group | n | Temperature (°C) | | | | |
|-------------------------|----|------------------|-------------------|-------------------|--------------------|--------------------|
| | | During Surgery | 1 h after Surgery | 6 h after Surgery | 24 h after Surgery | 48 h after Surgery |
| Nonoperated | 7 | 37.6 ± 0.1 | 37.6 ± 0.1 | 37.5 ± 0.1 | 37.8 ± 0.2 | 38.0 ± 0.2 |
| Sham-operated + vehicle | 6 | 37.4 ± 0.1 | 37.5 ± 0.2 | 37.5 ± 0.1 | 37.7 ± 0.1 | 37.8 ± 0.2 |
| TBI + vehicle | 10 | 37.6 ± 0.1 | 37.8 ± 0.2 | 37.4 ± 0.1 | 37.7 ± 0.1 | 37.6 ± 0.1 |
| TBI + fenofibrate | 6 | 37.5 ± 0.1 | 37.6 ± 0.2 | 37.4 ± 0.1 | 37.8 ± 0.2 | 38.0 ± 0.2 |
| TBI + simvastatin | 6 | 37.5 ± 0.1 | 37.6 ± 0.4 | 37.3 ± 0.1 | 37.6 ± 0.1 | 37.6 ± 0.1 |
| TBI + combination | 10 | 37.6 ± 0.1 | 37.9 ± 0.2 | 37.5 ± 0.1 | 37.7 ± 0.1 | 37.7 ± 0.1 |

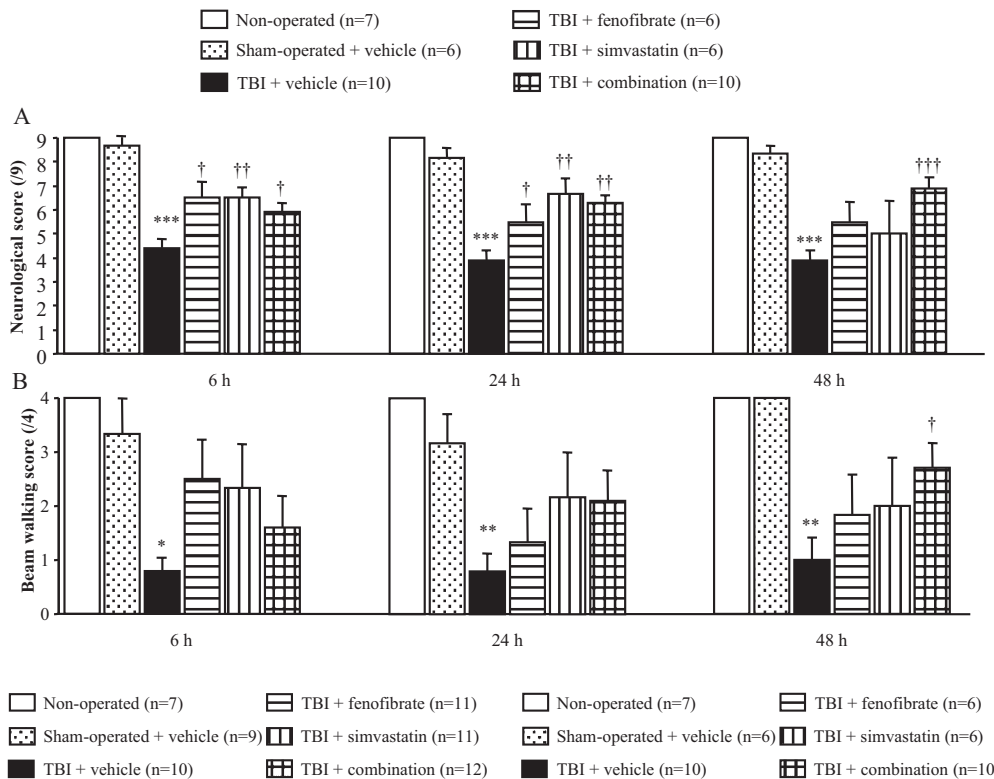


Fig. 3. Effects of fenofibrate, simvastatin, and their combination on the neurological score (A) and the beam-walking score (B) at 6, 24, and 48 h after TBI. Fifty micrograms per kilogram of fenofibrate, 37.5 mg/kg simvastatin, and their combination or its vehicle (water containing 0.5% methylcellulose) were administered p.o. at 1 and 6 h after TBI. A, rats that were treated with fenofibrate, simvastatin, or their combination showed an increase in the neurological scores at 6 h post-TBI that persisted at 24 h. At 48 h post-TBI, only the combination showed an improvement of the neurological score. B, both monotherapies and combination had no effect on the beam-walking scores at 6 and 24 h post-TBI. At 48 h postinjury, only rats receiving the combination showed an improvement on the beam-walking score. Data are presented as the mean ± S.E.M. Differences in neurological and beam-walking scores were determined by nonparametric Kruskal-Wallis analysis followed by a Mann-Whitney U test. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ versus sham-operated; †, $p < 0.05$, ††, $p < 0.01$, and †††, $p < 0.001$ versus TBI + vehicle.

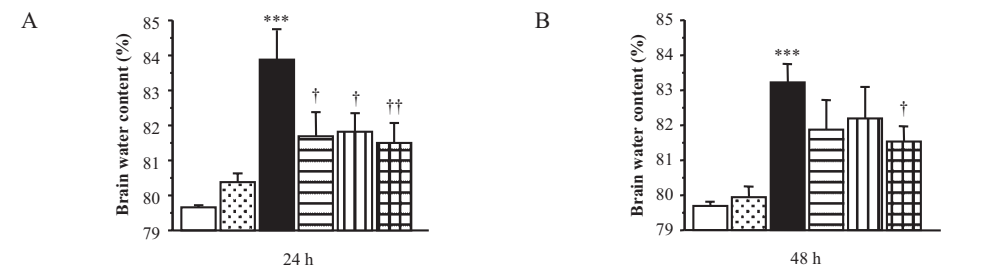


Fig. 4. Effect of fenofibrate, simvastatin, and their combination on the brain water content at 24 h (A) and 48 h (B) after TBI. Fifty micrograms per kilogram of fenofibrate, 37.5 mg/kg simvastatin, and their combination were administered p.o. at 1 and 6 h after TBI. A, at 24 h after injury, both the monotherapies and their combination reduced the brain water content. B, at 48 h post-TBI, only the combination reduced the brain water content. Data are presented as the mean ± S.E.M. Differences in brain water content was evaluated by one-way ANOVA with subsequent group comparisons by PLSD Fisher's test. ***, $p < 0.001$ versus sham-operated; †, $p < 0.05$ and ††, $p < 0.01$ versus TBI + vehicle.

$p < 0.01$), demonstrating a neurological deficit at 3 days post-TBI that persisted at 7 days (4.8 ± 0.4 , $p < 0.01$) (Fig. 5A). Both fenofibrate and simvastatin given alone did not reduce the neurological deficit at 3 and 7 days after injury. By contrast, rats treated with the combination showed an increase in the neurological score at 3 days (7.0 ± 0.8 , $p < 0.01$) that persisted at 7 days (7.2 ± 0.3 , $p < 0.01$).

TBI led to a decrease in the beam-walking score at 3 days (0.3 ± 0.3 versus 4.0 ± 0.0 for nonoperated, $p < 0.001$) that persisted at 7 days postinjury (0.9 ± 0.5 versus 4.0 ± 0.0 for nonoperated, $p < 0.01$), demonstrating a sustained post-trau-

matic deficit in motor coordination (Fig. 5B). Both fenofibrate and simvastatin did not increase the beam-walking score at 3 and 7 days post-TBI. By contrast, the combination promoted a recovery of motor coordination at 3 days (2.5 ± 0.7 , $p < 0.05$) that was maintained at 7 days postinjury (3.0 ± 0.4 , $p < 0.05$).

Effects of the Combination on Lesion Volume at 7 Days after TBI. TBI induced a brain lesion volume of $83.6 \pm 8.1 \text{ mm}^3$ that was reduced by treatment with the combination ($35.5 \pm 11.7 \text{ mm}^3$, $p < 0.05$), whereas both monotherapies did not reduce it (fenofibrate: $61.6 \pm 19.7 \text{ mm}^3$; simvastatin: $65.0 \pm 22.1 \text{ mm}^3$) (Fig. 6).

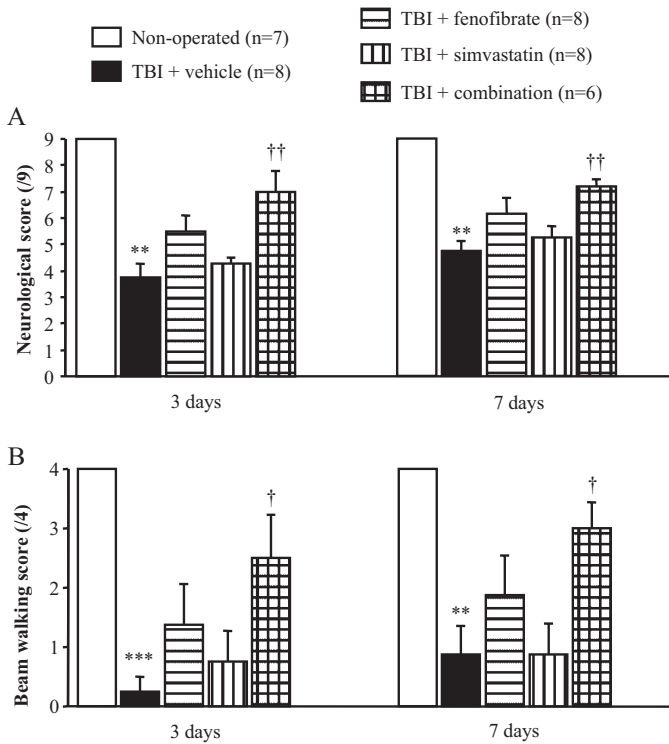


Fig. 5. Effect of fenofibrate, simvastatin, and their combination on the neurological (A) and beam-walking (B) scores 3 and 7 days after TBI. Fifty milligrams per kilogram of fenofibrate, 37.5 mg/kg simvastatin, and their combination were administered p.o. at 1 and 6 h after traumatic brain injury. Only the rats treated with the combination showed an increase in the neurological score and beam-walking score at 3 days that persisted at 7 days. Data are presented as the mean ± S.E.M. Differences in neurological and beam-walking scores were determined by nonparametric Kruskal-Wallis analysis followed by a Mann-Whitney *U* test. **, *p* < 0.01 and ***, *p* < 0.001 versus nonoperated; †, *p* < 0.05 and ††, *p* < 0.01 versus TBI + vehicle.

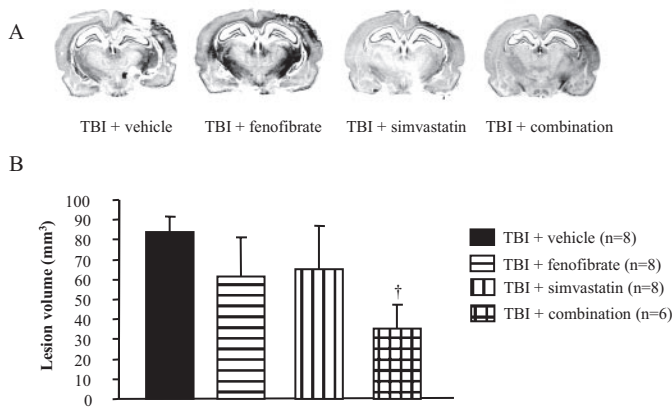


Fig. 6. A, representative brain sections stained with cresyl violet showing the lesion at the percussion epicenter from injured rats that received vehicle, fenofibrate, simvastatin, or their combination. B, effect of fenofibrate, simvastatin, and their combination on lesion volume induced by TBI. Fifty milligrams per kilogram of fenofibrate, 37.5 mg/kg simvastatin, and their combination were administered p.o. at 1 and 6 h after TBI. TBI induced a brain lesion volume that was reduced by treatment with the combination, whereas both monotherapies did not reduce it. Data are presented as the mean ± S.E.M. Differences in brain lesion volume was evaluated by one-way ANOVA with subsequent group comparisons by PLSD Fisher's test. †, *p* < 0.05 versus TBI + vehicle.

Effects of Delayed Treatment with the Combination on Neurological Function and Motor Coordination at 8, 24, and 48 h after TBI. Nonoperated rats had a global

neurological score of 9 (Fig. 7A). The neurological score of sham-operated rats was not different at 8, 24, and 48 h post-TBI. TBI led to a decrease in the neurological score at 8 h after injury ($4.2 \pm 0.2, p < 0.001$) that persisted at 24 h ($4.3 \pm 0.6, p < 0.001$) and 48 h after injury ($4.2 \pm 0.5, p < 0.001$). Treatment with simvastatin, administered at 3 and 8 h after TBI, improved the neurological score only at 48 h post-TBI ($5.9 \pm 0.5, p < 0.05$), whereas the combination increased the neurological score earlier than monotherapies. Indeed, the neurological recovery-promoting effect of the combined treatment was present at 8 h after injury ($6.1 \pm 0.4, p < 0.01$) and persisted at 24 h ($6.3 \pm 0.5, p < 0.05$) and 48 h post-TBI ($7.1 \pm 0.6, p < 0.01$).

Nonoperated rats had a beam-walking score of 4 (Fig. 7B). There was no difference between sham-operated and nonoperated rats at 8, 24, and 48 h after surgery. TBI led to a significant decrease in the beam-walking score at 8 h post-TBI ($0.4 \pm 0.3, p < 0.001$) that persisted at 24 h ($0.7 \pm 0.3, p < 0.001$) and 48 h after injury ($0.9 \pm 0.5, p < 0.01$). Both fenofibrate and simvastatin had no effect on the beam-walking score at each time point. By contrast, rats given the combination showed an improved beam-walking score at 24 h without reaching the statistical significance ($1.9 \pm 0.5, p = 0.06$), which became significant at 48 h post-TBI ($2.9 \pm 0.5, p < 0.05$).

Effects of Delayed Treatment with the Combination on Cerebral Edema at 48 h after TBI. The BWC of nonoperated and sham-operated rats was not different (79.7 ± 0.1 versus $79.9 \pm 0.2\%$) (Fig. 8). TBI led to an increase in the BWC 48 h after injury ($81.4 \pm 0.5\%, p < 0.05$), demonstrating a cerebral edema. However, delayed treatment with either monotherapies or combination had no effect on the BWC.

Discussion

There is evidence reporting that combined therapy with statins and fibrates is more effective than each drug alone in controlling atherogenic dyslipidemia (Koh et al., 2005). Previous data showed that long-term monotherapy with fenofibrate or simvastatin promoted neuroprotective and pleiotropic effects on inflammation in TBI (Besson et al., 2005; Chen et al., 2007a,b; Wang et al., 2007). Therefore, we studied the effects of combination of both drugs on post-traumatic consequences, and, to date, there was no information. In this study, to avoid risks, such as muscle toxicity associated with repeated dose of simvastatin (Law and Rudnicka, 2006) or fenofibrate (Davidson et al., 2007) and those associated with the combined therapy, we examined the effects of an acute post-treatment with the combination of fenofibrate and simvastatin on neurological deficits, brain edema, and brain lesion in a lateral fluid-percussion TBI model in rat.

Before studying the effects of the combination, we performed a dose-effect study for simvastatin, administered 1 and 6 h after injury, on post-traumatic neurological dysfunctions and brain edema, because only beneficial effects from long-term treatment with simvastatin were available in the literature. Because we previously showed that fenofibrate (administered at 1 and 6 h after TBI) exerted neuroprotective effects (Besson et al., 2005), this schedule was designed for simvastatin, keeping in mind its future use as combined therapy with fenofibrate. TBI induces sensorimotor and motor coordination dysfunctions that are evaluated by scores.

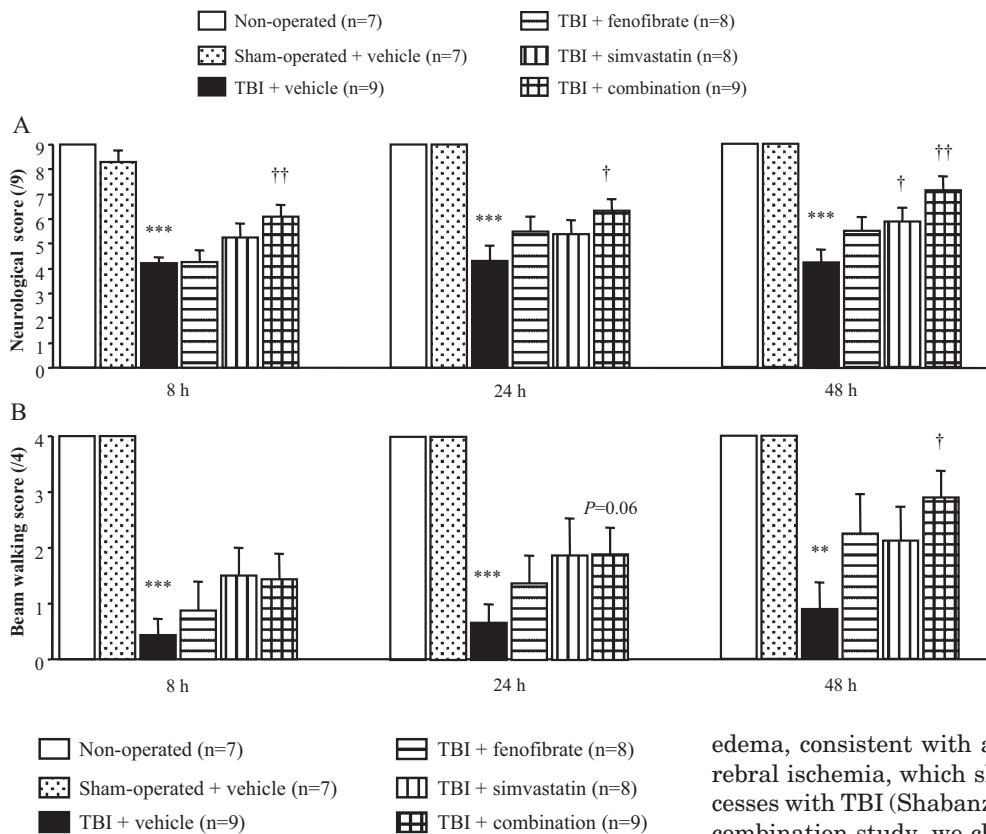


Fig. 7. Effect of delayed treatment with fenofibrate, simvastatin, and their combination on the neurological (A) and beam-walking (B) scores at 8, 24, and 48 h after TBI. Fifty milligrams per kilogram of fenofibrate, 37.5 mg/kg simvastatin, and their combination were administered p.o. at 3 and 8 h after TBI. A, fenofibrate had no effect at each time point. Simvastatin improved the neurological score only at 48 h post-TBI. The combination exerted the neurological recovery-promoting effect at 8 h after injury and persisted at 24 and 48 h post-TBI. B, both fenofibrate and simvastatin had no effect on the beam-walking score at each time point. Rats that were given the combination showed an improved beam-walking score at 24 h without reaching the statistical significance, which became significant at 48 h post-TBI. Data are presented as the mean \pm S.E.M. Differences in neurological and beam-walking scores were determined by nonparametric Kruskal-Wallis analysis followed by a Mann-Whitney *U* test. **, $p < 0.01$ and ***, $p < 0.001$ versus sham-operated; †, $p < 0.05$ and ††, $p < 0.01$ versus TBI + vehicle.

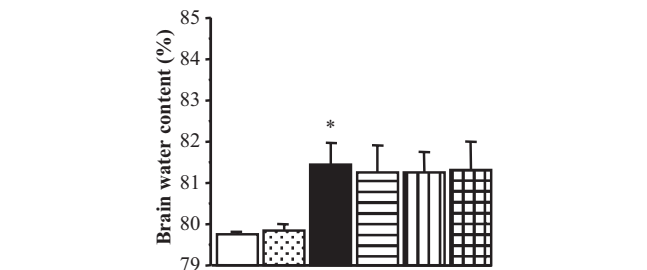


Fig. 8. Effect of delayed treatment with fenofibrate, simvastatin, and their combination on the brain water content 48 h after TBI. Fifty milligrams per kilogram of fenofibrate, 37.5 mg/kg simvastatin, or their combination were administered p.o. at 3 and 8 h after TBI. Delayed treatment with either monotherapies or combination had no effect on the brain water content. Data are presented as the mean \pm S.E.M. Differences in brain water content was evaluated by one-way ANOVA with subsequent group comparisons by PLSD Fisher's test. *, $p < 0.05$ versus sham-operated.

Neurological scores are clinically relevant endpoints, which are of particular importance because they are used in clinical trials of neuroprotective agents for TBI. In this study, we used a previously published neurological score to evaluate sensorimotor functions (Besson et al., 2003, 2005) and the beam-walking score as it has often been used to characterize motor coordination deficits associated with TBI (Wagner et al., 2007). Our data showed that 37.5 mg/kg simvastatin improved both neurological and beam-walking scores. These data are in agreement with studies showing that statins exert neurological recovery in experimental spinal cord injury (Pannu et al., 2005) and TBI models (Lu et al., 2004; Chen et al., 2007a; Wang et al., 2007). Our work is the first to study the effect of statin on post-traumatic edema. Our data showed that simvastatin at this dose reduced the brain

edema, consistent with a recent study of simvastatin in cerebral ischemia, which shares many pathophysiological processes with TBI (Shabanzadeh et al., 2005). Therefore, for the combination study, we chose the dose of 37.5 mg/kg for simvastatin and 50 mg/kg for fenofibrate, because we previously showed that this dose was neuroprotective in TBI (Besson et al., 2005).

Our data showed that treatment with fenofibrate or simvastatin or with combination significantly improved the sensorimotor functions at 6 and 24 h after injury. These beneficial effects were no longer present from 48 h until 7 days in monotherapy-treated rats. Our previous study showed that fenofibrate improved the sensorimotor functions at 3 and 7 days post-TBI, using the same protocol with additional administrations at 24, 48, and 72 h after TBI (Besson et al., 2005). Our present results coupled with these data demonstrated that prolonged monotherapy with fenofibrate is needed to maintain a long-term neurological improvement after TBI. In the literature, the neurological recovery-promoting effects of statins in TBI were seen with a pretreatment (Chen et al., 2007a; Wang et al., 2007) or with a long-term post-treatment (Wang et al., 2007). It is interesting to note that in the present study, the sensorimotor recovery was still present 48 h after injury in combination-treated animals and persisted at 7 days, demonstrating a long and sustained recovery induced by the combination.

Both monotherapies had no effect on the beam-walking scores at any time point post-TBI, whereas the combination therapy exhibited a motor coordination recovery that appeared at 48 h and persisted up to 7 days after injury. All of these data showed that combination of fenofibrate with simvastatin was more effective and exerted longer lasting effects on both sensorimotor and motor coordination deficits than each drug alone. Thus, combination may be a promising strategy to provide additional and longer beneficial effects after TBI, and as an acute treatment it will avoid potential side effects, such as muscle toxicity, which is associated with

repeated doses of either fibrates or statins (Law and Rudnicka, 2006; Davidson et al., 2007).

In the clinical setting, cerebral edema is a cause of morbidity and death in traumatized patients because it may increase intracranial pressure, decrease cerebral perfusion, and lead to brain herniation (Unterberg et al., 2004). In this experimental model of TBI, edema develops early after trauma, increases within the first 15 h, and persists up to 48 h after injury (Bareyre et al., 1997). Our data showed that monotherapies and combination therapy reduced the post-traumatic brain edema by 70% at 24 h. More importantly, the combination still reduced 50% of the brain edema at 48 h after injury, exhibiting a potent long-lasting antiedematous effect, whereas fenofibrate and simvastatin, when given alone, had no more effect at this time point. Our data suggested that two acute administrations of the combination induce a prolonged reduction of brain edema.

TBI results in cerebral tissue lesion measured at 7 days after injury. The combination reduced the brain lesion volume, whereas the monotherapies failed to reduce it, demonstrating that the combined therapy is more efficient. Monotherapy with statins or fibrates have been demonstrated to decrease brain lesion with a repeated dose regimen. Indeed, we previously showed that 50 mg/kg fenofibrate, using the same protocol with additional treatments at 24, 48, and 72 h after TBI, restricted the lesion at 7 days after injury. However, high-dose fenofibrate increased the brain lesion, indicating that a high dose associated with a long-term treatment with fenofibrate may induce adverse effects on cell death (Besson et al., 2005). In addition, long-term pretreatment with lovastatin reduced brain damage volume after TBI (Chen et al., 2007a). In this study, short-term post-treatment with combination of fenofibrate and simvastatin synergistically reduced brain lesion volume, whereas monotherapies alone had no effect. Acute treatment with combination versus repeated with monotherapy, what is the best option? The pathophysiological situation of traumatized patients should be taken into account. Indeed, these patients, as in this model of TBI (Moinard et al., 2005), are shown to be hypercatabolic. Therefore, from a nutritional and metabolic point of view, an acute treatment with combination should be the best choice for the treatment of TBI.

Our data showed that the combination, administered at 1 and 6 h post-TBI, decreased the neurological deficits, brain edema, and lesion volume. Furthermore, we examined whether a delayed treatment, which is more relevant to the clinical reality, could improve the neurological outcome as well as reduce the brain edema. When treatments were administered later (i.e., 3 and 8 h after injury), the combination reduced both the neurological and motor coordination deficits at 8, 24, and 48 h, whereas fenofibrate and simvastatin did not show any effect on both scores. Each treatment had no effect on brain edema at 48 h post-TBI, demonstrating that for the antiedematous effect, the therapeutic window is less than 3 h after brain trauma.

The present study is the first to evaluate acute combined treatment of fenofibrate and simvastatin in brain injury and to show a long-lasting effect of the combination on neurological deficits, brain edema, and brain lesion. Simvastatin and fenofibrate are both widely used in clinical practice as lipid-lowering drugs for treatment of dyslipidemia. Several studies reported that combined therapy with statins and fibrates is

more effective in controlling atherogenic dyslipidemia in patients with combined hyperlipidemia than each drug alone (Koh et al., 2008). Although mechanisms of action of each drug alone have been well described in the literature (Stepień et al., 2005; Bordet et al., 2006), the mechanisms of the synergism of the combination are not completely clear. Laboratory studies demonstrated synergistic effects of statins combined with fibrates. Indeed, statins inhibit the Rho-signaling pathway and activate PPAR α (Martin et al., 2001), and acute anti-inflammatory properties of statins involve PPAR α via inhibition of the protein kinase C-signaling pathway (Paumelle et al., 2006). Fibrates and statins synergistically increase the transcriptional activities of PPAR α /retinoid X receptor α and decrease the transactivation of NF- κ B (Inoue et al., 2002). It has recently been demonstrated that statins, through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, increase 15*d*-prostaglandin J2 levels leading to the activation of PPAR γ , another subtype of PPAR family (Yano et al., 2007). Moreover, in vitro, statins suppressed lipopolysaccharide-induced expression of monocyte chemoattractant protein-1 and tumor necrosis factor α and activation of two transcription factors, NF- κ B and activator protein-1, through both PPAR α - and PPAR γ -dependent pathways (Yano et al., 2007). Therefore, future studies are needed to further elucidate the mechanisms involved in the synergism of the combination in TBI.

Evidence that fenofibrate combined with simvastatin promote long-lasting beneficial effects on neurological recovery, brain edema, and lesion provides a strong basis for the use of this combination for the treatment of TBI consequences. Last but not the least, combined oral administration of fenofibrate and simvastatin is devoid of any possible pharmaceutical or intestinal absorption interactions after a single dose (Penn et al., 2006). Therefore, translating this strategy to a clinical context may potentially have important therapeutic significance.

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