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Research Report

Efficacy and safety profile of the carotenoid trans sodium crocetinate administered to rabbits following multiple infarct ischemic strokes: A combination therapy study with tissue plasminogen activator

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ABSTRACT

Trans sodium crocetinate (TSC) is a synthetic small-molecule antioxidant that has the ability to enhance oxygen diffusion to hypoxic tissue. Because TSC is a promising drug candidate to treat acute ischemic stroke (AIS), we tested the hypothesis that TSC may be neuroprotective following cerebral ischemia using a rabbit small clot embolic stroke model (RSCEM) using clinical rating scores as the endpoint. TSC or saline was administered IV following the injection of small blood clots into the brain vasculature. Behavior was measured 24 h following embolization in order to calculate the effective stroke dose (P_{50}) that produces neurological deficits in 50% of the rabbits. A treatment is considered beneficial if it significantly increases the P_{50} compared to control. TSC (0.25 mg/kg) given 5 or 60 min following embolization significantly ($p<0.05$) increased P_{50} values by 104% and 181%; but not when given 3 h post-embolization (48% increase, $p>0.05$). tPA (3.3 mg/kg) produced a significant increase in P_{50} when given 1, but not 3 h following embolization. In combination studies, when TSC was administered 1 h and tPA was given either 1 or 3 h following embolization, the group P_{50} values were increased by 291% and 140%, respectively. In addition, TSC plus tPA administered 3 h following embolization significantly ($p<0.05$) increased the group P_{50} value by 90%. There were no significant effects ($p>0.05$) of either TSC alone or TSC administered in combination with tPA on intracerebral hemorrhage incidence. This study suggests that TSC may be used for the treatment of AIS either alone or when administered before or concomitant with tPA to improve clinical rating scores with a therapeutic window for TSC therapy up to 3 h in rabbits. Moreover, it appears that TSC can be administered with tPA, since the combination did not result in any significant change in intracerebral hemorrhage incidence.

1. Introduction

We have focused on investigating the neuroprotective effects of a variety of natural products including baicalein, chlorogenic acid and fisetin on ischemia-induced clinical deficits using the rabbit small clot embolic stroke model (RSCEM) (Lapchak, 2007; Lapchak et al., 2007a; Maher et al., 2007), mainly because many natural products can affect multiple

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steps in the ischemic cascade (Dirnagl et al., 1999; Lapchak and Araujo, 2007a,b). Many natural products hold promise as a possible treatment for acute ischemic stroke (AIS), which is estimated to affect more than 700,000 patients each year in the USA and 15 million patients worldwide (Ingall, 2004; Lapchak and Araujo, 2007a) primarily because they are effective antioxidants (dos Santos et al., 2006; Nijveldt et al., 2001; Yen et al., 2005) and antioxidants have been shown to be useful in preclinical and clinical stroke studies (Belayev et al., 2002; Floyd, 1999; Lapchak, 2007; Lapchak and Araujo, 2007a; Lapchak and Zivin, 2009; Watanabe et al., 2008; Yamaguchi et al., 1998). It has been reported that many natural products have multiple mechanisms of action, which increase their potential usefulness to treat AIS (see Masella et al., 2005; Nijveldt et al., 2001; Woodman and Chan, 2004 for reviews).

Recently, Gainer (2008) described an interesting molecule designated as trans-sodium crocetinate (TSC), which is a carotenoid related to naturally produced crocetin that can be purified from Saffron (Abe and Saito, 2000). TSC is a pure trans-isomer, with a novel hypothesized mechanism of action (Gainer, 2008). Even though TSC has the ability to act as a free radical scavenger, especially hydroxyl radicals (Stennett et al., 2007), its primary mechanism of action is hypothesized to be related to its ability to enhance oxygen diffusion between erythrocytes and tissues (Gainer, 2008), in essence, creating an oxygen gradient in favor of tissue. Preclinical studies in a rodent ischemia models have shown that TSC can reduce infarct volume measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining, an effect that may be directly related to increased brain $P0_2$ levels in ischemic tissue (see Manabe et al., 2007). However, even though TSC can reduce infarct volume measured, the authors did not show that the "neuroprotection" at the cellular level would translate into a clinical benefit using a clinically relevant endpoint such as behavior; that is the main purpose of the present study.

In 1996 the FDA approved the thrombolytic, tissue plasminogen activator (tPA) for the treatment of stroke (NINDS rt-PA Trial, 1995). To date, tPA remains the only approved therapy for stroke. Since there is considerable need to identify new or adjunctive therapies to be used in combination with tPA, we sought to determine whether TSC would be useful as a therapeutic agent to treat AIS resulting from small clot embolization. For this, we used the RSCEM, which is a useful tool and possibly a predictor of effective treatments that may eventually translate into efficacy in human clinical trials, because the primary cause of injury resulting in behavioral deficits is an embolism that causes cerebral ischemia (Lapchak et al., 2002; Lapchak et al., 2004a,d; Lapchak and Araujo, 2007a; Lapchak et al., 2007b). Moreover, the primary endpoint used when assessing treatment efficacy in the RSCEM is behavioral functional, which is based upon motor function components of the National Institute of Health Stroke Scale (NIHSS) for stroke in humans (Broderick et al., 2000; Clark et al., 2000).

Thus, in the present study we evaluated the pharmacological effects of TSC in the RSCEM as a basis for the further clinical development of this compound. The RSCEM (Lapchak et al., 2002, 2004a,d, 2007a) is produced by the injection of blood clots into the cerebral vasculature. A wide range of clots doses are injected in order to generate both normal and abnormal animals with various behavioral deficits, which can be measured quantitatively using a simple dichotomous rating scale (Lapchak et al., 2002, 2004a,d, 2007a; Lyden et al., 2000). Using the RSCEM, the present study tested the hypothesis that TSC would be useful to attenuate embolism-induced behavioral deficits. Moreover, since optimal doses of tPA do not eliminate all brain damage, even though tPA does increase cerebral reperfusion (reviewed in Lapchak, 2002a), we investigated the interaction between tPA and TSC to determine whether there are any positive or synergistic interactions when the drugs are combined. The experimental design used for combination studies was based upon that documented by Lyden et al. (2000), an important study that previously used the quantal analysis technique to demonstrate synergistic effects of combination therapy following middle cerebral artery occlusion in rodents. Moreover, the design was based upon the modification by Lapchak (2007). Since tPA can cause intracerebral hemorrhage (ICH) in patients and embolized rabbits (Albers et al., 2000; Lapchak, 2002b, 2004c; Lapchak and Araujo, 2007a,b; Lapchak, 2009; Lyden et al., 1987, 1990; Lyden and Zivin, 1993), we also determined the consequences of combining TSC with tPA on ICH incidence in embolized rabbits.

2. Results

2.1. *TSC dose–response analysis*

In this series of studies, we determined the effects of administration of TSC (0.03–0.5 mg/kg) on behavioral function measured 24 h following embolization. In this study, TSC at doses of 0.125–0.25 mg/kg improved behavior, but only the 0.20 and 0.25 mg/kg doses improved behavior and significantly increased the P_{50} values to 2.14±0.44 mg ($n=20$; $p=0.028$) and 2.24±0.37 mg ($n=20$; $p=0.0079$), respectively compared to the vehicle control group which had a P_{50} value of 1.10±0.22 mg ($n=26$). TSC when dosed at 0.125 mg/kg had a P_{50} value of 2.10 ±0.51 mg ($n=20$; p=0.057). Fig. 1A shows a graphical representation of the raw data that is superimposed on the theoretical statistically fit quantal analysis curves for vehicle-treated and TSC (0.25 mg/kg)-treated rabbits. Fig. 1B provides the dose–response analysis curve profile for TSC in rabbits following embolic strokes.

2.2. *TSC therapeutic window analysis*

For therapeutic window analysis, a TSC dose of 0.25 mg/kg was used based upon the data provided in Fig. 1. For this and subsequent series of experiments, we used a cumulative control group because we have previously documented that the baseline P_{50} value for embolized rabbits is quite consistent over time and between experiments (Lapchak et al., 2002, 2004a,d, 2007a) and the use of the cumulative control allows us to reduce the usage of large numbers of rabbits. Fig. 2A shows a graphical representation of quantal analysis curves for the study. TSC (0.25 mg/kg) was beneficial when given 1 h [$P_{50}=2.84\pm1.01$ mg ($n=24$, $p<0.05$)], but not 3 h [$P_{50}=1.49$ ±0.45 mg ($n=24$, $p<0.05$)] following embolization, compared to a cumulative vehicle control group, which had a P_{50} value of 1.01±0.23 mg ($n=34$). Fig. 2B provides the therapeutic window

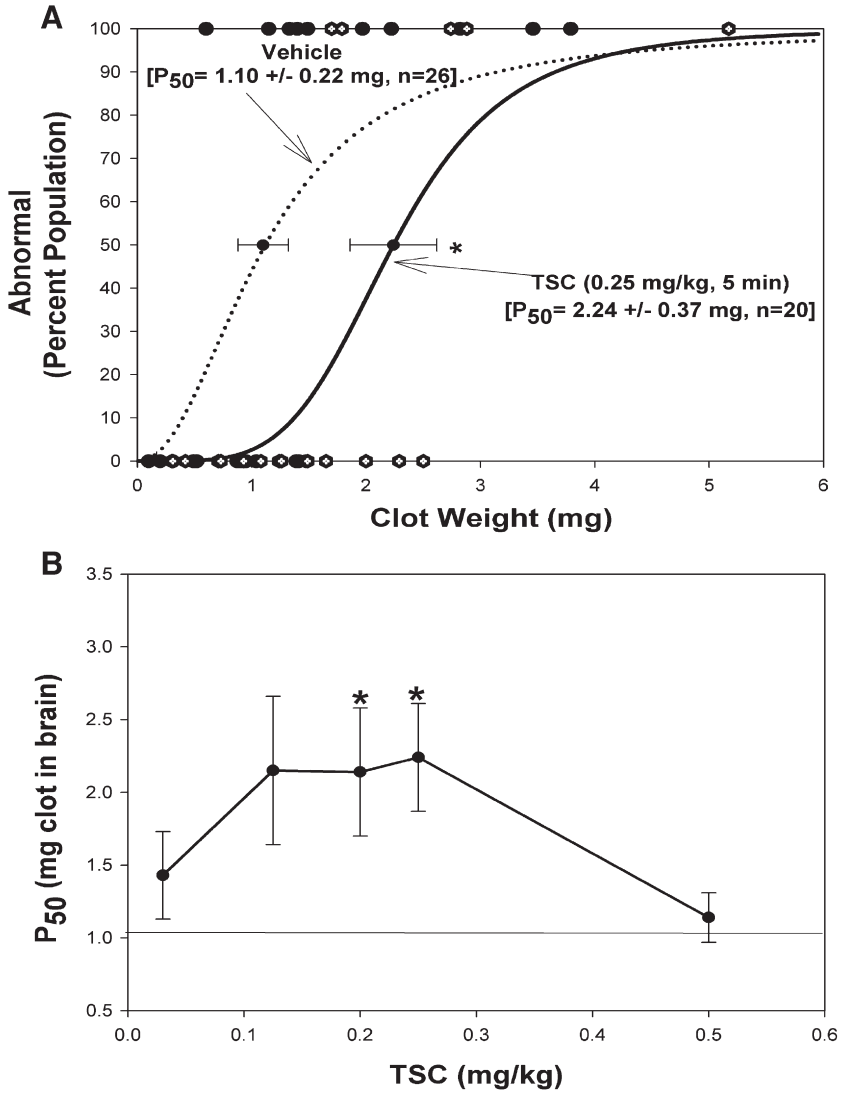

Fig. 1 – (A) Quantal curves: Effect of TSC (0.25 mg/kg, solid line) given 5 min post-embolization on behavior measured following embolic strokes compared to control (dotted line). For the superimposed graphs, normal animals are plotted on the y-axis at 0 and abnormal animals are plotted at 100. The figure shows that there is positive correlation between the data [circles (vehicle) or open-center hexagons (TSC)] and the statistically fit sigmoidal quantal curve. TSC significantly (*$p<0.05$) improved behavior compared to control. (B) Dose–response curve: Behavior (P_{50} in mg clot in brain) as a function of TSC dose (mg/kg) administered 5 min post-embolization. Results are shown as mean ± SEM. Significantly different compared to the vehicle-treated control (*$p<0.05$).

profile for TSC in rabbits following embolic strokes when given 1 and 3 h following embolization. TSC was shown to be effective when administered 5 min and 1 h following embolization, but TSC did not significantly improve behavior when administered 3 h after embolization.

2.3. *TSC combination studies*

2.3.1. *TSC and tPA with a 1 h delay*

In this series of studies, we examined the effect of administration of TSC in combination with tPA to determine whether the combination would have an additive or synergistic effect on behavioral outcome. In previous studies, we have found that combination therapy could increase the therapeutic window for thrombolytics (Lapchak et al., 2002, 2004b; Lapchak, 2006, 2007). Using an add-on design, quantal analysis curves for tPA and the TSC/tPA combination were constructed. tPA significantly improved behavior and increased the group P_{50} value of 2.48 ± 0.17 mg ($n=21$, $p<0.05$) compared to the vehicle control group, which had a P_{50} value of 1.01 ± 0.23 mg ($n=34$). In combination studies, when both TSC and tPA were administered 1 h following embolization, the group P_{50} value measured 24 h following embolization was 3.95 ± 0.73 mg ($n=26$), a P_{50} that was significantly different from control ($p<0.05$). There was a trend for a synergistic effect of the drug combination that did not reach statistical significance compared to TSC ($p=0.372$) or tPA ($p=0.087$).

2.3.2. *TSC (1 h delay) and tPA (3 h delay)*

Fig. 3 provides a graphical representation of the effects of tPA on behavior and the group P_{50} value when tPA was given 3 h following embolization. tPA did not significantly improve behavior ($p>0.05$) resulting in a group P_{50} value of 1.00 ± 0.56 mg

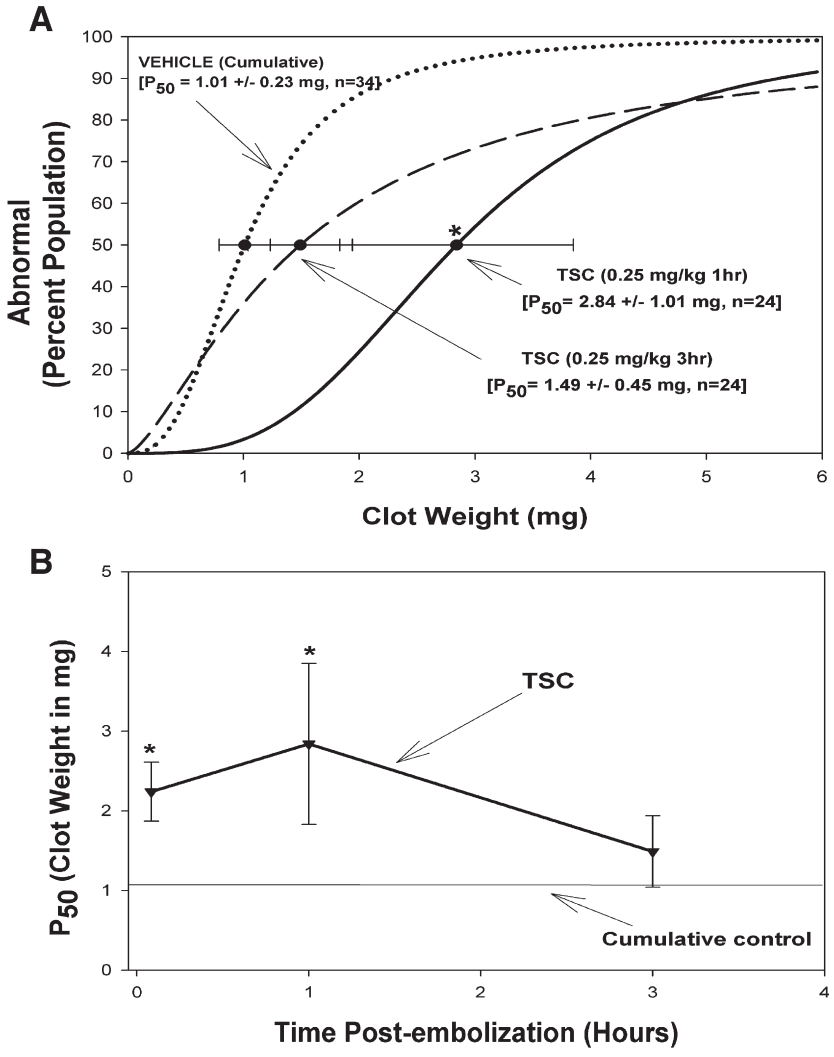

Fig. 2 – (A) Quantal curves: Abnormal rabbits as a function of clot weight measured in brain. Results are shown as mean ± SEM for the number of rabbits in each group (*n*). The curve labeled Vehicle (dotted line) shows that 50% of rabbits with a clot dose (P_{50} value) of 1.01 ± 0.23 mg (*n* = 34) are Abnormal. TSC (0.25 mg/kg) treatment 1 h post-embolization (solid line) significantly increased the P_{50} value to 2.84 ± 1.01 mg (*n* = 24. **p* < 0.05). TSC 3 h post-embolization (dashed line) increased the P_{50} value to 1.49 ± 0.45 mg (*n* = 24, *p* > 0.05). (B) Therapeutic window curve: Behavior (P_{50} in mg clot in brain) as a function of time after embolization (in hours). TSC (0.25 mg/kg) was administered IV in order to determine the maximum therapeutic window for behavioral improvement. Results are shown as mean ± SEM. Significantly different compared to the vehicle-treated control (p* < 0.05).**

(*n* = 27) compared to the vehicle cumulative control group, which had a P_{50} value of 1.01 ± 0.23 mg (*n* = 34). In combination studies, when TSC was administered 1 h following embolization and tPA was given 3 h following embolization, the group P_{50} value was 2.43 ± 0.24 mg (*n* = 20), a P_{50} that was significantly different from control (*p* < 0.05).

2.4. *TSC combination studies: TSC and tPA (3 h delays)*

In previous studies, we have shown that combining a pharmacological with tPA at extended therapeutic windows following embolization could produce a synergistic effect or extend the therapeutic window for the thrombolytic (Lapchak and Zivin, 2003; Lapchak et al., 2004b; Lapchak, 2006, 2007). Here we tested the hypothesis that combining TSC and tPA at maximally effective doses (0.25 and 3.3 mg/kg, respectively), but at a time point (3 h post-embolization) when neither drug alone produces a significant behavioral improvement, may produce synergy and significantly improve clinical rating scores. This test was important and scientifically interesting because both TSC and tPA have similar effects, that is to increase oxygen diffusion to hypoxic tissue, but both have very different mechanisms of action. Fig. 4 shows the results of the combination-treated group compared to monotherapy with each drug. Neither tPA, nor TSC improved behavior (*p* > 0.05) resulting in a group P_{50} value of 1.00 ± 0.56 mg (*n* = 27) and 1.49 ± 0.45 mg (*n* = 24) compared to the vehicle cumulative control group, which had a P_{50} value of 1.01 ± 0.23 mg (*n* = 34). The combination of TSC and tPA, when administered 3 h following embolization had a P_{50} value of 1.92 ± 0.31 mg (*n* = 22, *p* < 0.05).

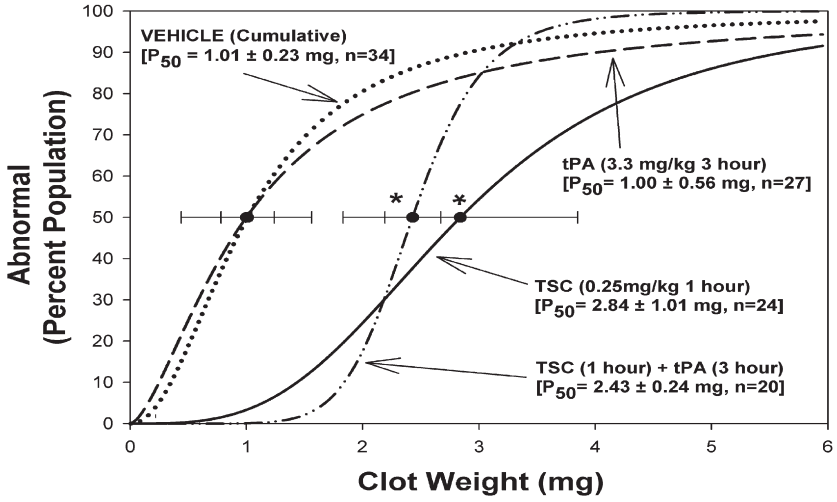

Fig. 3 – Combination analysis: Abnormal rabbits as a function of clot weight measured in brain. Results are shown as mean ± SEM for the number of rabbits in each group (n). TSC (0.25 mg/kg, solid line) treatment 1 h post-embolization significantly (*$p<0.05$) increased P_{50} values compared to control (dotted line). tPA was ineffective when given 3 h following embolization (dashed line). The combination of TSC (1 hour) plus tPA (3 h), shown as a dashed-dotted line, significantly (*$p<0.05$) improved behavior and increased P_{50} values compared to the vehicle control group (dotted line).

2.5. *Effect of monotherapy or combination therapy on hemorrhage rate*

Since there is literature showing that thrombolytics can significantly increase brain hemorrhage in patients (NINDS rt-PA Study, 1995) and rabbits following embolic strokes (Albers et al., 2000; Lapchak, 2002b; Lapchak et al., 2004c; Lapchak and Araujo, 2007a,b; Lapchak, 2009; Lyden et al., 1987, 1990; Lyden and Zivin, 1993), we determined the consequences of tPA administration on hemorrhage rate in embolized rabbits in the presence or absence of TSC treatment. For this, prior to the measurement of the radioactive content of Co^{57} microspheres in brain, brain tissue was examined for ICH according to previously published criteria (Lapchak et al., 2004c; Lapchak, 2009). Similar to those studies, we found that there was a low incidence of ICH in the control embolized group (Table 1A–C). In this study, we found that 5.8% of control rabbits had ICH compared to either 11.1% or 14.2% for tPA administered 1 or 3 h following administration, respectively. There was a low incidence of ICH (0–4.2%) in the groups of rabbits that received TSC alone and this was not significantly ($p>0.05$) affected by administering TSC with tPA (see Table 1A–C).

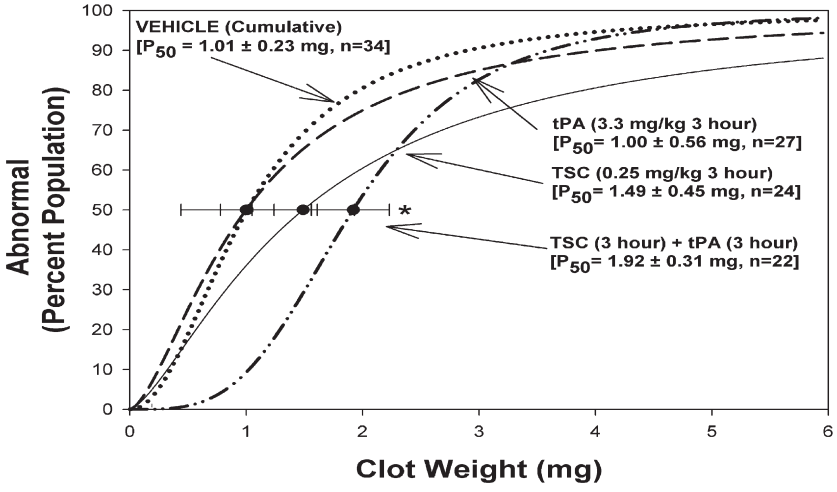

Fig. 4 – Delayed combination analysis: Abnormal rabbits as a function of clot weight measured in brain. Results are shown as mean ± SEM for the number of rabbits in each group (n). The curve labeled Vehicle control (dotted line) shows that 50% of rabbits with a clot dose (P_{50} value) of 1.01 ± 0.23 mg ($n=34$) are Abnormal. TSC (0.25 mg/kg, solid line) treatment 3 h post-embolization had a P_{50} value of 1.49 ± 0.45 mg ($n=24$). tPA (3.3 mg/kg, dashed line) treatment 3 h post-embolization had a P_{50} value of 1.00 ± 0.56 mg ($n=27$). The combination of TSC (3 h) plus tPA (3 h) shown as a dashed-dotted line resulted in a significant increase (*$p<0.05$) of the P_{50} value to 1.92 ± 0.31 mg ($n=22$) compared to control (dotted line).

Table 1 – Effect of TSC on hemorrhage rate in embolized rabbits.

	Treatment group			
Measure	Control	TSC	tPA	TSC + tPA
(A) TSC 1 h treatment group + tPA 1 h treatment group				
ICH rate	2/34 (5.8%)	1/24 (4.2%)	3/21 (14.2%)	2/26 (7.7%)
p-value	NA	1.00	0.38	1.00
(B) TSC 1 h treatment group + tPA 3 h treatment group				
ICH rate	2/34 (5.8%)	1/24 (4.2%)	3/27 (11.1%)	2/20 (10.0%)
p-value	NA	1.00	0.65	0.63
(C) TSC 3 h treatment group + tPA 3 h treatment group				
ICH rate	2/34 (5.8%)	0/24 (0%)	3/27 (11.1%)	2/22 (9.09%)
p value	NA	0.51	0.65	1.00

Statistical significance was determined using a two-tailed chi-square test. All *p*-values provided are for comparisons made between the Treatment group and the Control group.

3. Discussion

In this study, we determined whether TSC, a multifunctional compound that is an antioxidant and has the ability to enhance oxygen diffusion between erythrocytes and tissues, would affect behavioral outcome of embolized rabbits. The present study shows that TSC administration significantly improves clinical rating scores when administered as a monotherapy within 1 h of embolization. Because the primary endpoint used in quantal analysis is behavioral function, which is mainly composed of motor functions, it appears that post-embolization TSC administration effectively improves motor function measured 24 h following embolization. Moreover, the study shows that TSC may be administered safely in combination with the thrombolytic tPA and that combination therapy produces a significant behavioral improvement in embolized rabbits. The data suggest that TSC may also extend the treatment window for tPA to at least 3 h, a time at which tPA alone is ineffective in this animal model.

In this study we relied on a validated behavioral outcome measure using a quantal analysis technique, to study the effects of monotherapy and combination therapy with TSC since the RSCEM is a multiple infarct ischemia model. Because the RSCEM utilizes a suspension of small clots containing Co^{57} microspheres to produce a stroke, embolization results in multiple small infarct cores and areas of ischemia. The Co^{57} microspheres are used to determine the content of blood clots in brain following embolization. Thus, because of the use of the gamma-producing radiolabel and the presence of multiple small infarct sites, the RSCEM cannot be used to measure infarct volume with standard TTC staining procedures as was done in the rodent model and reviewed in Gainer (2008). Moreover, this study is a logical extension of a previous study that has already documented that TSC can decrease infarct volume in rodents (see Manabe et al., 2007), a study that did not demonstrate that TSC could improve an important clinically relevant outcome such as behavioral function.

The TSC dose–response analysis curve presented in Fig. 1B shows that TSC has a narrow therapeutic window and the curve in an inverted hyperbolic curve. We found that doses between 0.10 and 0.25 mg/kg produced behavioral increases, but only 0.20–0.25 mg/kg doses produced statistically significant behavioral improvements in embolized rabbits. However, the highest dose studied was not deleterious, just inactive. The reason for this lack of effect of the highest dose studied is not known.

Recent pharmacological studies with TSC have shown that the compound may be useful to treat hemorrhagic shock (Giassi et al., 2001, 2002; Roy et al., 1998; Stennett et al., 2006, 2007), traumatic brain injury (Stern et al., 2002) and possibly stroke. Manabe et al. (2007) have also demonstrated TSC efficacy in a rodent 3 vessel occlusion stroke model. The Manabe study, which is reviewed in Gainer (2008) indicates that TSC can significantly reduce infarct volume following ischemia and also increased PO_2 levels. Okonkwo et al. (2003) also studied the pharmacology of TSC in rats by measuring PO_2 levels in brain tissue. The authors concluded that TSC significantly increased brain tissue oxygen delivery, which is consistent with the wealth of knowledge concerning crocetin and TSC (Gainer, 2008; Giassi et al., 2001, 2002; Okonkwo et al., 2003; Seyde et al., 1986; Sheehan et al., 2009; Stennett et al., 2006). Thus, the main mechanism of action of TSC may be related to its ability to increases oxygen delivery by increasing the diffusivity of oxygen through plasma. However, carotenoids such as TSC also have the ability to be free radical scavengers (Gainer, 2008; Giaccio, 2004) and TSC has been shown to decrease the production of cytokines such as TNFα and interleukin-10 (Stennett and Gainer, 2004). Taken together, it appears that the beneficial effects of TSC may be due to intervention as various points within the ischemic cascade and its inherent ability to increase oxygen availability to ischemic brain tissue.

We hypothesize that the 1–3 h therapeutic window observed in the RSCEM is equivalent to approximately 3 and 9 h after a stroke in patients hours in a stroke patient because of the following considerations based upon recently accumulated correlative data from preclinical studies using the RSCEM and clinical trials in which treatments were found to be effective. In the RSCEM, tPA effectively improves behavior when given 1-1.5 h following embolization and in patients the therapeutic window for tPA is along the order of 3–4.5 h after stroke onset (Del Zoppo et al., 2009; Lansberg et al., 2009; Lapchak, 2002a; Lapchak et al., 2004a; Saver et al., 2009). Moreover, the free radical trapping compound Radicut (Edaravone) has a 3 h therapeutic window in the RSCEM (Lapchak and Zivin, 2009) and is used clinically (in Japan) up to 72 h following a stroke. Although Japanese clinicians use Edaravone within 72 h of a stroke, there is little clinical evidence to support its use (EAISG, 2003; Higashi et al., 2006; Kitagawa, 2006; Suda et al., 2007; Tanahashi and Fukuuchi, 2002; Watanabe et al., 2008). We have also shown that transcranial laser therapy (TLT) has a long therapeutic window, up to 6 h in the preclinical setting (Lapchak et al., 2004d, 2007b). The recent clinical trials of TLT have documented efficacy in patients when treated within 24 h of a stroke (Lampl et al., 2007; Zivin et al., 2009). Thus, there appears to be a 2 to 4-fold differential between the observed times for effectiveness of treatments

in the rabbit model compared to AIS patients, excluding the results with Edaravone noted above.

In conclusion, we have demonstrated considerable efficacy for TSC in the rabbit embolic stroke model and have also shown that it is safe to administer TSC in combination with tPA. Our studies suggest that TSC may either be used as a monotherapy or in combination with current FDA-approved thrombolytic therapy to improve clinical outcome in acute ischemic stroke patients. Moreover, TSC is safe to administer in combination with tPA.

4. Experimental procedures

4.1. *Animals and animal welfare*

Male New Zealand white rabbits weighing 2 to 2.5 kg were purchased from Rabbit Source Farms, Ramona, CA. Rabbits were supplied food (alfalfa cubes) and water ad libitum while under quarantine in an enriched environment for at least 5 days prior to experimental use. Surgery was done in a sterile controlled environment with a room temperature between 22.8 and 23.2 °C. Institutional Animal Care and Use Committee (IACUC) approved the surgical and treatment procedures used in this study. Extreme care was used throughout the study to minimize pain and discomfort. Per the IACUC-approved protocol, rabbits were euthanized if they were in pain, showed extreme discomfort, or if they were unable to reach food or water.

4.2. *Surgery and embolization*

Surgical procedures were done as described previously (Lapchak et al., 2002, 2004a,d, 2007a). Rabbits were anesthetized with halothane via a face-mask, 5% in 3 l/min at induction, and 3% in 3 l/min as a maintenance dose. The right internal carotid artery was exposed, and the external carotid artery and the common carotid artery were ligated. A Becton, Dickinson and Company (B-D) plastic catheter oriented toward the brain was inserted into the common carotid and secured with ligatures. The incision was closed around the catheter so that the distal end was accessible outside. The catheter was filled with 0.2 ml of heparinized saline (33 units/ml) and plugged with injection caps. The animals were allowed to recover from anesthesia for at least 2 h before embolization.

Blood is drawn from one or more donor rabbits and allowed to clot for 3 h at 37 °C. The large blood clots are then suspended in phosphate-buffered saline pH 7.4 (PBS) with 0.1% bovine serum albumin (BSA) and Polytron-generated fragments are sequentially passed through metal screens and nylon filters. Small-sized blood clots were suspended in PBS containing 0.1% bovine serum albumin and labeled with ^{57}Co containing New England Nuclear Inc. (NEN)-Trac microspheres prior to embolization. An aliquot of the solution was removed for the determination of specific activity. For embolization, fully awake rabbits are placed in restrainers and 1 ml of a clot particle suspension is injected through an indwelling carotid catheter positioned toward the brain. The catheter is then flushed with 5 ml of sterile saline. Rabbits are fully awake during the embolization procedure and they are self-maintaining (i.e.: they do not require artificial respiration or other external support). This allows for immediate observation of the effects of embolization on behavior at the time of clot injection and thereafter. After the embolization process was completed, the catheter was heat ligated close to the neck. Animals that died prior to treatment were excluded from the study and those that died prior to sacrifice but received full treatment(s) were included in this study. The surviving animals were euthanized 24 h post-embolization with 1–1.5 ml of Beuthanasia-D via the marginal ear vein.

4.3. *Clinical rating score behavioral and quantal dose–response analysis*

The use of clinical rating scores and quantal analysis is a statistical method to determine how a large population of stroke "patients" in this case, rabbits, will respond to a treatment and is an appropriate primary endpoint to use when a treatment is being developed to support a clinical trial. To evaluate the quantitative relationship between clot dose in brain and behavioral deficits or clinical scores, logistic sigmoidal quantal analysis curves were fitted to the dose–response data as originally described by Waud (1972) and thereafter (Lapchak et al., 2002, 2004a,d, 2007a). A wide range of clot doses were injected in this study in order to produce a spectrum of behaviorally normal to abnormal animals. In the absence of a neuroprotective treatment regimen, small numbers of microclots cause no grossly apparent neurologic dysfunction. However, when large numbers of microclots are injected and enter the brain vasculature, they invariably caused encephalopathy and sometimes death. In this study, less than 5% of animals died due to embolization, but in the event that embolization did result in a rapid death, there was a positive correlation with a high clot dose measured in brain and the animal was represented as an abnormal (or dead) on the quantal analysis curve if the rabbit received treatment. Abnormal rabbits with encephalopathy include those with one or more of the following symptoms: ataxia, leaning, circling, lethargy, nystagmus, loss of balance, loss of limb/facial sensation and occasionally, paraplegia. Using a simple dichotomous rating system, with a reproducible composite result and low inter-rater variability (<5%), each animal was rated as either behaviorally normal or abnormal by a naive-observer (S. Nunez). Using quantal analysis, we are to detect behavioral changes following pharmacological intervention. Using the system, we are unable to detect small changes in improvement over the duration of the study, which may be considered a type-two error because of reduced power to detect small changes. However, the model and analysis are well suited to detect robust changes following pharmacological intervention.

A separate curve was generated for each treatment condition and a statistically significant increase in the P_{50} value or the amount of microclots in brain that produce neurologic dysfunction in 50% of a group of animals compared to control is indicative of a behavioral improvement and neuroprotection.

4.4. *Drug treatment*

For test substance administration, rabbits were placed in a Plexiglas restrainer for the duration of the treatment.

4.4.1. *Dose–response analysis*

For dose–response curve analysis, 5 min following embolization, rabbits were given a bolus IV injection of vehicle or TSC (0.03–0.50 mg/kg) over 1 min using the marginal ear vein at a dose volume of 0.22 ml/kg.

4.4.2. *Therapeutic window analysis*

For therapeutic window analysis, 1–3 h following embolization, rabbits were given a bolus IV injection of vehicle or TSC (0.25 mg/kg) over 1 min using the marginal ear vein at a dose volume of 0.22 ml/kg.

4.4.3. *Combination studies*

For thrombolytic studies, tPA (3.3 mg/kg) was given IV, 1 or 3 h post-embolization, with 20% as a bolus injection over 1 min, followed by the remaining 80% infused over 30 min. Clinical grade tPA lyophilized in 50 mg configurations, containing 50 mg tPA (29 million IU), 1.7 mg L-arginine, 0.5 g phosphoric acid and less than 4 mg Polysorbate 80, that was then reconstituted with sterile water (1 mg/ml) was purchased from Genentech, Inc. (South San Francisco, CA) for the studies. TSC (0.25 mg/kg) was also given 1 or 3 h following embolization.

4.5. *Power and statistical analysis*

Power analysis of quantal dose–response curves indicates that, assuming $\alpha = .05$ and $\beta = .90$, a coefficient of variation of 15% and a difference between means of 20%, a sample size of 14 animals are required per group. The behavioral data are presented as P_{50} (Mean ± SEM) in mg clot for the number of rabbits in each group (n). P_{50} values were calculated using an iterative curve fitting algorithm described previously (Waud, 1972; Zivin and Waud, 1992). They were analyzed for significance using the t-test with SigmaStat 3.5. For statistical analysis of ICH incidence or rate, we used two-sided chi-square analysis analyzed using MedCalc Version 9.4.1.0. as described previously (Lapchak et al., 2008; Lapchak, 2009; Lapchak and Han, 2009).

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