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Single-dose ebselen does not afford sustained neuroprotection to rats subjected to severe focal cerebral ischemia

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Abstract

Oxygen free radicals have been involved in the pathophysiology of cerebral ischemia, especially after spontaneous or thrombolytic reperfusion. In this study with rats, we have combined a severe focal ischemic insult (2 h) and a prolonged reperfusion time (7 days) to assess the possible sustained neuroprotective effect of ebselen (10 or 100 mg/kg), a small, lipophilic organoselenium compound which mimics glutathione peroxidase. Parietal cortical perfusion was measured by laser-Doppler flowmetry, and focal cerebral ischemia was carried out by the intraluminal thread method. We have measured plasma selenium levels, brain reduced glutathione levels, as a marker of oxidative stress, and infarct volume associated with cerebral ischemia. Focal ischemia did not alter reduced glutathione levels, while 60 min reperfusion following ischemia induced a significant ($P<0.05$) decrease in reduced glutathione levels of the ipsilateral hemisphere. Pretreatment with ebselen, which induced significant ($P<0.05$) increase in plasma selenium levels, did not significantly alter the decrease in reduced glutathione levels. The ischemic insult induced 30% mortality on average, with deaths always occurring within 12–48 h. Surviving rats suffered up to 25% body weight loss 1 week after the ischemic insult. Infarct volumes were 26.8 ± 4.7% of the hemisphere in placebo-treated rats, 26.6 ± 3.6% in 10 mg/kg ebselen-treated rats, and 25.6 ± 6.4% in 100 mg/kg ebselen-treated rats (not significantly different). Single-dose administration of ebselen does not reduce the size of brain infarct resulting from severe focal cerebral ischemia in rats. In contrast to previous studies with relatively earlier endpoints, we have delayed the measurement of infarct volume to 1 week after the ischemic insult.

Keywords: Ebselen; Focal ischemia; Oxidative stress; Cerebral infarct; (Rat)

1. Introduction

Oxygen free radicals have been involved in the pathophysiology of cerebral ischemia, especially after spontaneous or thrombolytic reperfusion. Although technically difficult, free radical production can be directly measured in ischemic cerebral tissue (Mori et al., 1999; Peters et al., 1998). Other studies provide indirect evidence based on the irreversible damaging effects of free radicals against cellular macromolecules (Oliver et al., 1990; Polidori et al., 1998), as well as on the use of transgenic and knockout animals with altered levels of oxidant and antioxidant enzymes (Chan, 2001).

Both, free radical scavenging molecules (e.g. ascorbic acid, α-tocopherol, etc.), and coupled antioxidant enzymatic activities (i.e. superoxide dismutase, catalase and glutathione peroxidase) have detoxifying functions during normal cellular activity (Matés, 2000). However, sudden reoxygenation during post-ischemic reperfusion induces overproduction of reactive oxygen species, inactivation of antioxidant activities and consumption of antioxidants, which leads to brain injury by means of direct damage to cellular macromolecules, namely lipids, proteins and nucleic acids, and activation of specific signal transduction pathways of oxidative stress (Chan, 2001).

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Antioxidant therapy in chronic and acute neurologic disease is based in the use of different classes of endogenous, exogenous and even synthetic antioxidants (Delanty and Dichter, 2000). The main limitations of endogenous enzymatic antioxidants are their large size, which limits cell permeability, short circulating half-life, antigenicity, and expense (Cuzzocrea et al., 2001). This probably has lead to conflicting results when native and modified superoxide dismutases have been used in experimental cerebral ischemia (Chan, 2001). On the other hand, in the use of nonenzymatic or low molecular weight antioxidants, a good degree of blood–brain barrier penetrance should be achieved (Gilgun-Sherki et al., 2001).

Ebselen is a small, lipophilic organoselenium compound which mimics glutathione peroxidase, reacts with peroxynitrite, and can inhibit prooxidant enzymes (Parnham and Sies, 2000). Previous preclinical studies have reported neuroprotective effects of ebselen reducing infarct volume in rodents after transient or permanent focal cerebral ischemia. It should be noted that early endpoints for evaluation of neuroprotection (Dawson et al., 1995; Imai et al., 2001, 2003; Takasago et al., 1997; Lapchak and Zivin, 2003) or relatively mild ischemic insults (Namura et al., 2001) were employed. In our study, we have combined a severe focal ischemic insult (2 h) and a more prolonged reperfusion time (7 days) to assess the possible sustained neuroprotective effect of ebselen. We have measured hemodynamic parameters (brain perfusion and arterial blood pressure) and metabolic parameters (blood pH, pCO_2, pO_2, and glucose) to monitor the ischemia–reperfusion process and assess possible effects of ebselen administration. After the ischemic insult, we have measured brain reduced glutathione levels, as a marker of oxidative stress, in addition to infarct volume associated with cerebral ischemia.

2. Materials and methods

Experiments were performed in accordance with the guideline from the Council of the European Economic Community (86/609/EEC, Article 5, Appendix II), promulgated by the Spanish legislation on March 14, 1988 (R.D. 223/1988). Sixty nine male Wistar rats (Charles River Laboratories España) weighing 300–350 g were used.

2.1. Transient focal cerebral ischemia induction

Anesthesia was induced by intramuscular injection of diazepam (Valium, Roche, 7 mg/kg body weight), ketamine (Ketolar, Parke-Davis, 70 mg/kg body weight) and atropine (B Braun Medical, 0.15 mg/kg body weight). Rats were mounted in the prone position on a stereotaxic instrument (Kopf Instruments). After a cranial midline incision, a burr hole (1 mm in diameter) was made with a microdrill 2 mm posterior and 3.5 mm right to bregma. A truncated 21G needle was fixed on the hole with two miniature screws and dental cement. Once the wound was sutured, the animal recovered from anesthesia and fasted for 24 h.

To induce focal cerebral ischemia, the rats were anesthetized with 4% halothane (Fluothane, Zeneca) in a mixture of 70% nitrous oxide and 30% oxygen by means of endotracheal intubation and assisted ventilation (Harvard 683 rodent ventilator). Anesthesia was maintained during the operative procedures with 0.5–1% halothane in the same gas mixture. The flexible plastic tip (0.5 mm in diameter, PF319, Perimed) of a master laser-Doppler probe (PF418, Perimed) was introduced through the cranial 21G needle until the tip of the probe gently contacted the brain surface, thus measuring the parietal cortical perfusion in the territory of the middle cerebral artery by means of a laser-Doppler flowmeter (PF4001 Master, Perimed). Core temperature was measured and maintained around 37.5 °C by means of a rectal temperature probe (YSI 402, Yellow Spring Instrument) connected to a thermometer and a feedback-regulated heating blanket (HB101/2, Letica). The left femoral artery was cannulated with a polyethylene catheter (Braun OD 0.9 mm) for arterial blood pressure measuring (1290C pressure transducer, Hewlett-Packard, connected to a blood pressure monitor, Stoelting) and blood sampling (for pH, pCO_2, pO_2, and glucose determination). Laser-Doppler cortical perfusion, body temperature and arterial blood pressure signals were digitalized (PF472 A/D converter, Perimed) and sent to an IBM® PC compatible computer for continuous recording, storage and later analysis.

Middle cerebral artery occlusion was induced by using the intraluminal thread technique (Longa et al., 1989). Briefly, the right common, external, and internal carotid arteries were identified through a ventral cervical midline incision. After electrocoagulation and cutting of its branches, the external carotid artery was ligated and cut 2 mm distal to the bifurcation. The pterygopalatine artery was ligated, and microclips were placed across both the common and internal carotid arteries. A 3 cm length of 4-0 monofilament nylon suture (Harvard), its tip rounded by heating near a soldering iron, was introduced into the external carotid artery through a puncture. After tightening a silk suture around the external carotid artery and the intraluminal nylon suture to prevent bleeding, the microclips were removed and the nylon suture was gently advanced into the internal carotid artery and circle of Willis until the origin of the middle cerebral artery was reached (approximately 20 mm from the carotid bifurcation). This was indicated by both slight resistance to nylon suture advance and sudden fall in cortical perfusion as measured by the laser-Doppler probe. For reperfusion after 2 h of ischemia, the intraluminal suture was carefully removed. In chronic experiments, the neck and femoral incisions were closed, and the rats were allowed to survive for 7 days with free access to food and water.

2.2. Ebselen administration: experimental groups

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, Sigma) was suspended in olive oil and administered by gavage through a feeding needle (Fine Science Tools) 1 h before the ischemic insult. Only olive oil was administered to placebo-treated (control) animals. Acute experiments were carried out to assess the effects of ischemia, reperfusion and ebselen on brain reduced glutathione content as a marker of oxidative stress. For this purpose, placebo-treated rats were sacrificed after 120 min ischemia ($n=6$) or 120 min ischemia followed by 60 min reperfusion ($n=4$). Ebselen-treated rats (10 or 100 mg/kg body weight) were sacrificed after 120 min ischemia followed by 60 min reperfusion ($n=5$ and $n=4$, respectively). Sham-operated rats were also included to determine basal brain reduced glutathione levels ($n=5$). Chronic experiments were carried out to assess the effects of ebselen on infarct volume associated with transient cerebral ischemia. For this purpose, placebo- or ebselen-treated rats (10 or 100 mg/kg body weight) were subjected to 120 min ischemia, reperfused and sacrificed after a survival period of 1 week ($n=14$, $n=12$ and $n=9$, respectively). A separate group of rats ($n=10$) was used to determine plasma selenium levels before and after oral ebselen administration (10 or 100 mg/kg body weight).

2.3. Brain reduced glutathione measurement

Rats were sacrificed by intracardiac injection of KCl (UCB Pharma, 75 mg/kg) while under deep anesthesia. The brain was removed and a 2-mm-thick coronal section (6–8 mm from anterior pole) was obtained by means of a tissue slicer (Stoelting). The two hemispheric parts of the slice (ipsilateral and contralateral to the ischemic insult) were separated, flash frozen in liquid N_2 and stored at −80 °C until use. For reduced glutathione content determination, tissue samples were homogenised in perchloric acid 2% (containing EDTA 1 mM). Reduced glutathione was assayed by high performance liquid chromatography (Reed et al., 1980). Briefly, 0.2 ml sample (acidic supernatant after centrifugation) was treated with 0.04 ml iodoacetic acid (100 mM), pH adjusted to 8.5–9 with KOH (2 M), and allowed to react with an excess of 1-fluor-2,4-dinitrobenzene overnight at room temperature. After centrifugation, the supernatant was separated through a 5 μm 3-Spherisorb-NH2, 250 × 4.6 mm column with an increasing gradient of sodium acetate in methanol/water (4:1, v/v). Calibration curves were run daily. Protein content was determined by the method of Lowry.

2.4. Plasma selenium measurement

Since ebselen is a selenoorganic compound, we estimated plasma levels of ebselen by measuring plasma selenium levels. Heparinized blood samples were taken 1, 2, 3, 4, 24, 72 and 144 h following ebselen administration. All samples were immediately centrifuged, plasma removed and frozen at −20 °C until selenium analysis.

Selenium was measured by electrothermal atomic absorption spectrometry (ET-AAS 1100B, Perkin Elmer) with a deuterium correction, after addition of $Pd/Mg(NO_3)_2$ as modified matrix and an appropriate dilution (Alegría et al., 1996). Accuracy was checked by means of a reference material (Seronorm Trace Elements Serum Level 1 from Sero AS).

2.5. Cerebral infarct measurement

Brain infarction was determined by the 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) vital staining method (Bederson et al., 1986) slightly modified. Rats were anesthetized by intramuscular injection of ketolar (150 mg/kg), and sacrificed by intracardiac injection of KCl (UCB Pharma, 75 mg/kg). The brain was removed and six 2-mm-thick coronal sections were obtained by means of a tissue slicer (Stoelting). Brain slices were immersed in a 2% solution of TTC in saline solution at 37 °C for 15 min, after which slices were fixed in 10% phosphate-buffered (pH 7.4) formalin overnight. Since macrophage infiltration occurs during maturation of the infarct, we obtained an accurate index of brain injury area 1 week after the transient ischemic insult by shortening TTC incubation time to half the originally described time (Bederson et al., 1986) in order to maintain good contrast between damaged and healthy tissue. The border between infarcted and noninfarcted tissue was outlined with an image analysis system (Kontron Elektronik), and the area of infarction was measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral hemisphere. The volume of infarction was calculated by

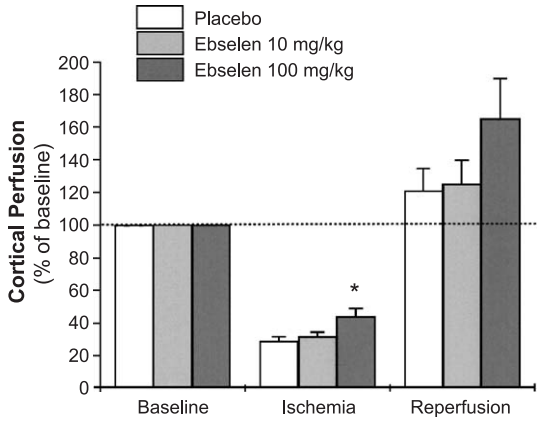

Fig. 1. Cortical perfusion measured before, during and after transient focal cerebral ischemia in rats treated with placebo, 10 mg/kg ebselen, or 100 mg/kg ebselen. Data are mean ± S.E.M. *Significantly different from placebo ($P<0.01$).

Table 1
Arterial blood pH, gasses, and glucose measured before, during, and after focal cerebral ischemia

	Placebo	Ebselen (10 mg/kg)	Ebselen (100 mg/kg)
Ph			
Preischemia	7.38 ± 0.01 (24)	7.43 ± 0.01[a] (18)	7.47 ± 0.01[a] (12)
Ischemia	7.32 ± 0.01[b] (23)	7.32 ± 0.03[b] (18)	7.35 ± 0.02[b] (12)
Reperfusion	7.32 ± 0.02[c](13)	7.32 ± 0.02[c] (5)	7.35 ± 0.05[b] (3)
pCO_2 (mm Hg)			
Preischemia	47 ± 2 (24)	33 ± 1[a] (18)	34 ± 1[a] (12)
Ischemia	53 ± 2 (23)	40 ± 2[a,b] (18)	43 ± 3[c,d] (12)
Reperfusion	50 ± 5 (13)	37 ± 3 (5)	41 ± 5 (4)
pO_2 (mm Hg)			
Preischemia	104 ± 4 (24)	113 ± 5 (18)	119 ± 7 (12)
Ischemia	86 ± 5[c](23)	112 ± 6[a] (18)	113 ± 4[a] (12)
Reperfusion	92 ± 6 (13)	117 ± 12 (5)	124 ± 10[d] (4)
Glucose (mg/dl)			
Preischemia	105 ± 6 (24)	86 ± 4[d] (18)	88 ± 4 (12)
Ischemia	106 ± 7 (22)	74 ± 3[a] (18)	78 ± 4[a] (12)
Reperfusion	105 ± 14 (12)	74 ± 18 (5)	80 ± 10 (4)

Data are mean ± S.E.M. from (*n*) determinations.
[a] Significantly different from placebo ($P<0.01$).
[b] Significantly different from preischemia ($P<0.01$).
[c] Significantly different from preischemia ($P<0.05$).
[d] Significantly different from placebo ($P<0.05$).

integration of the lesion areas at the six measured levels of the brain.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. Student's *t*-test or analysis of variance followed by Dunnett's test (multiple comparison versus a control) were used. A value of $P<0.05$ was considered statistically significant.

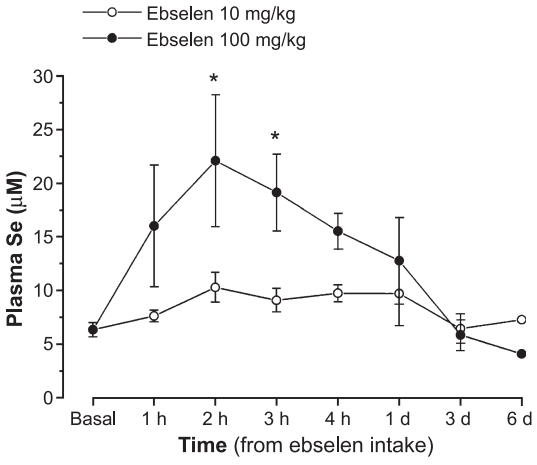

Fig. 2. Plasma selenium levels in rats before and following treatment with 10 mg/kg ebselen, or 100 mg/kg ebselen. Data are mean ± S.E.M. *Significantly different from basal levels ($P<0.01$).

3. Results

Introduction of the intraluminal nylon suture produced an immediate and maintained fall in cortical perfusion below 30% of baseline on average in placebo-treated rats (Fig. 1). Cortical perfusion reduction in rats treated with 10 mg/kg ebselen was similar to that in placebo-treated rats, while in rats given 100 mg/kg ebselen, the reduction in cortical perfusion was significantly ($P<0.01$) less than in placebo-treated rats (Fig. 1). On filament withdrawal, cortical perfusion recovered values slightly above baseline in both placebo- and ebselen-treated rats. Although perfusion values tended to be higher in rats treated with 100 mg/kg ebselen than in placebo-treated rats, this did not reach statistical significance due to the variability of cortical perfusion on middle cerebral artery recanalization (Fig. 1). Although a transient reactive hypertensive response followed suture introduction, mean arterial blood pressure did not significantly change before, during and after the ischemic insult (86 ± 3, 92 ± 4, and 82 ± 4 mm Hg, respectively).

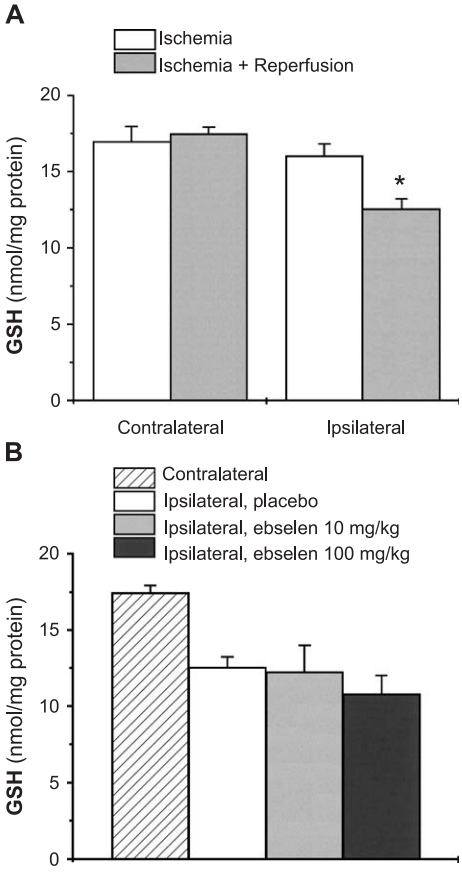

Fig. 3. Brain reduced glutathione content in the contralateral and ipsilateral brain hemispheres. (A) Rats subjected only to 120 min focal ischemia, or to 120 min ischemia followed by 60 min reperfusion. *Significantly different from contralateral ($P<0.05$). (B) Rats subjected to ischemia + reperfusion, and treated with placebo, 10 mg/kg ebselen, or 100 mg/kg ebselen. Value in the contralateral hemisphere is included as a control. No significant differences were found between ipsilateral hemispheres of ebselen- and placebo-treated rats. Data are mean ± S.E.M.

Arterial blood pH, gasses, and glucose measured 10 min before ischemia onset, 90 min after ischemia onset, and 20 min after reperfusion onset (i.e. 140 min after ischemia onset) are shown in Table 1 for the three experimental groups. Slight acidosis developed, with pH significantly reduced during ischemia ($P<0.01$) and reperfusion ($P<0.05$). Slight hypoxia also developed during ischemia ($P<0.05$) and recovered on reperfusion. Glucose and pCO_2 levels did not significantly change during or after the ischemic insult. On the other hand, ebselen treatment showed some significant alkalotic, hypocapnic, hyperoxic, and hypoglycemic effects (see Table 1 for details).

Basal endogenous plasma selenium levels were 6.35 ± 1.63 μM. Following oral ebselen administration, plasma selenium levels increased and reached peak values by 2 h. As shown in Fig. 2, plasma selenium levels were significantly ($P<0.01$) above basal levels 2 and 3 h after 100 mg/kg ebselen administration.

Reduced glutathione levels were 15.3 ± 0.5 nmol/mg protein in brain tissue from sham-operated rats. Focal ischemia did not alter reduced glutathione levels, which were not significantly different in cerebral hemispheres ipsilateral and contralateral to the ischemic insult (Fig. 3A). By contrast, 60 min reperfusion following 120 min ischemia induced a significant ($P<0.05$) decrease in reduced glutathione levels of the ipsilateral hemisphere, but not in the contralateral hemisphere (Fig. 3A). Pretreatment with ebselen (either 10 or 100 mg/kg) did not significantly alter the decrease in reduced glutathione levels induced by ischemia–reperfusion in the ipsilateral hemisphere of placebo-treated rats (Fig. 3B).

Some rats included in the group destined to survive 1 week for infarct volume determination died 12–48 h after ischemia. Mortality rates were 6 out of 14 in placebo-treated rats, 2 out of 12 in rats treated with 10 mg/kg ebselen, and 3 out of 9 in rats receiving 100 mg/kg ebselen. Surviving rats suffered body weight loss to $75 \pm 4\%$ of preischemic weight in placebo-treated group, to $85 \pm 4\%$ in 10 mg/kg ebselen-treated group, and to $87 \pm 6\%$ in 100 mg/kg ebselen-treated group. No significant difference was found when body weight losses in the three groups were compared.

One week after the ischemic insult, brain slices showed TTC-delineated areas of infarcted tissue, which affected both cortical and striatal structures of the ipsilateral hemisphere and were maximal at 6–8 mm from the anterior pole. Infarct areas measured in each of the six brain slices of placebo- and ebselen-treated (10 and 100 mg/kg) rats are shown in Table 2. Integrated infarct volumes were $26.8 \pm 4.7\%$ of the hemisphere in placebo-treated rats, $26.6 \pm 3.6\%$ in 10 mg/kg ebselen-treated rats, and $25.6 \pm 6.4\%$ in 100 mg/kg ebselen-treated rats. Volume of infarct in ebselen-treated rats was not significantly different from volume in placebo-treated rats.

4. Discussion

Our study in rats shows that a single dose of the antioxidant ebselen does not reduce the size of the infarct resulting from 2 h of transient focal cerebral ischemia and measured 1 week after the ischemic insult. Some previous studies have reported neuroprotective efficacy of ebselen histologically assessed 4 h after transient focal ischemia (Dawson et al., 1995), 24 h after permanent focal ischemia (Takasago et al., 1997), and 24 h after hypoxia/ischemia (Knollema et al., 1996). These results prompted the Japanese Ebselen Study Group to carry out clinical trials showing beneficial effects in acute ischemic stroke (Yamaguchi et al., 1998) and acute middle cerebral artery occlusion (Ogawa et al., 1999). Further studies in rodents and rabbits have reported neuroprotective efficacy of an intravenous formulation of ebselen again at relatively early endpoints (4–24 h) after focal cerebral ischemia (Imai et al., 2001, 2003; Lapchak and Zivin, 2003) and antineuroapoptotic effects of ebselen after relatively mild (30 min) focal ischemia (Namura et al., 2001). In this study, we aimed to extend the assessment of the neuroprotective effects of ebselen to a more delayed endpoint after a severe ischemic insult in order to test the possible beneficial effects of the antioxidant against postponed brain damage (Corbett and Nurse, 1998).

Although ebselen has been claimed to be a safe drug in clinical trials (Yamaguchi et al., 1998), we closely checked for undesirable effects of the antioxidant by monitoring blood pH, gasses, and glucose. On one hand, typical metabolic acidosis developed as a consequence of the ischemic insult. On the other hand, some mild alkalotic, hypocapnic, hyperoxic, and hypoglycemic effects were associated to ebselen treatment. These are potentially beneficial effects as opposed to suggested worsening effects

Table 2
Areas of infarct in coronal brain slices and integrated infarct volume in placebo- and ebselen-treated rats

	Infarct area (%)						Infarct volume (%)	*n*
	Distance (mm)	2–4	4–6	6–8	8–10	10–12	12–14	
Placebo		n.d.	29 ± 4	45 ± 6	43 ± 8	26 ± 8	9 ± 5	26.8 ± 4.7	8
Ebselen, 10 mg/kg		4 ± 2	27 ± 6	47 ± 5	46 ± 6	26 ± 5	5 ± 3	26.6 ± 3.6	10
Ebselen, 100 mg/kg		5 ± 4	37 ± 6	46 ± 6	38 ± 10	18 ± 8	2 ± 2	25.6 ± 6.4	6

Data are mean ± S.E.M. from *n* determinations. Infarct areas and volumes are expressed as % of the hemislice and hemisphere, respectively (n.d., not detected). Distance is expressed in mm from the anterior pole of the brain.

like acidosis, hypercapnia, hypoxia and hyperglycemia. Although acidosis is a well established feature of ischemic brain tissue, the historically negative view of acidosis is being challenged in the last few years by evidence suggesting a rather beneficial role (Tombaugh and Sapolsky, 1993). Far from considering lactic acidosis as a detrimental factor, recent studies have demonstrated lactate as an excellent aerobic energy substrate in the brain, and possibly a crucial one immediately post-ischemia (Schurr, 2002). Hypercapnia is not considered an important aggravating condition in cerebral ischemia, as even strongly elevated pCO_2 (300 mm Hg) only marginally affected ischemia-induced changes in brain intracellular signalling processes (Kurihara et al., 2004). Normobaric hyperoxia could be beneficial during transient stroke. However, increased oxygenation theoretically may increase oxygen free-radical injury, particularly during reperfusion. It has been recently reported that hyperoxia treatment during focal cerebral ischemia–reperfusion in the rat is neuroprotective, and does not increase oxidative stress (Singhal et al., 2002). However, hyperoxic levels attained is that study (pO_2 around 480 mm Hg) were by far higher than slight hyperoxia attributed to ebselen treatment in our experiments. Finally, hyperglycemia is clearly associated with worsening of post-ischemic brain injury in animal models and in humans (Wass and Lanier, 1996; Kagansky et al., 2001). Instead of lactic acidosis, the mechanism underlying hyperglycemic damage seems to be a short-lived elevation in the release of glucocorticoids (Schurr, 2002). Taken together, we think that the magnitude of the metabolic changes induced by ebselen treatment was pathologically irrelevant, as has been previously reported by others (Imai et al., 2001; Knollema et al., 1996; Takasago et al., 1997). In fact, they did not contribute to improve or impair post-ischemic outcome in our study.

Relatively high doses of ebselen, in a single dosage oral regime, have been used in this study. Ebselen was administered once 1 h before ischemia on the basis of previously published papers reporting short-term (up to 24 h) protective effects with similar monodose treatments (Dawson et al., 1995; Knollema et al., 1996; Kondoh et al., 1999; Lapchak and Zivin, 2003). Oral administration to 24 h fasted rats would theoretically allow time for intestinal absorption and blood–brain barrier penetration by the time the burst of oxidative stress takes place on reperfusion. Shortly after ebselen administration, plasma selenium levels increase over endogenous selenium levels. Once basal selenium subtracted, estimated plasma ebselen concentration in high-dose treated rats reaches values well above 4–5 μM, which have been reported as protective in both myocardial (Hoshida et al., 1994) and cerebral (Takasago et al., 1997) ischemia. Thus, following gavage administration, ebselen is rapidly absorbed by the gastrointestinal tract, and reaches potentially therapeutic plasma levels during the ischemic insult and early reperfusion. Although we did not measure brain ebselen, it has been reported that, due to its small size and lipophilicity, ebselen penetrates the blood–brain barrier and brain levels of the drug were 21% of plasma levels 1 h after administration to rats (Imai et al., 2001). With regard to pharmacokinetics, ebselen easily suffers isoselenazolone ring-opening to form a selenosulphide, which in turn becomes methylated or glucuronidated. In any case, the selenium moiety does not become bioavailable. Therefore, ebselen does not enter the body pool of selenium, but it is metabolised and excreted, which may explain its lack of toxicity (Parnham and Sies, 2000).

The value of continuous monitoring of cortical perfusion in the intraluminal suture model of focal cerebral ischemia has been previously stressed (Schmid-Elsaesser et al., 1998). In our study, this monitoring allowed not only to exclude rats showing inadequate middle cerebral artery occlusion or reperfusion, but also to check for effects of ebselen treatment on cortical microcirculation. Thus, every rat included in the study suffered a rather severe ischemic insult with perfusion values around 30% of baseline, in a penumbral measurement point, and 120-min duration. In a previous study using the same ischemia model (Salom et al., 2000), we have reported that the neuroprotective effects of the nitric oxide (NO) donor spermine/NO can be at least partially mediated by improvement of intraischemic brain perfusion. In the present study, rats treated with high-dose ebselen showed a modest 15% improvement in cortical perfusion during middle cerebral artery occlusion compared to placebo and low-dose ebselen-treated rats.

Oxidative stress can be directly detected by measuring production of reactive oxygen species, or indirectly deduced by measuring, for example, irreversible damage in biological macromolecules, or consumption of low molecular weight endogenous antioxidants involved in antioxidant defence. Reduced glutathione is a substrate for glutathione peroxidase to inactivate reactive oxygen species (Matés, 2000). In our study, brain reduced glutathione levels were not altered by ischemia, but were lowered when reperfusion follows the ischemic insult. This shows that, in our transient ischemia model, oxidative stress develops only when reoxygenation of ischemic tissue increases the formation of reactive oxygen species and overcomes reduced glutathione regeneration. Our experiments also show that ebselen administration does not modify the decrease in brain reduced glutathione content induced by ischemia–reperfusion. This is in line with the mechanism of action of ebselen, a glutathione peroxidase mimic, that also utilises reduced glutathione to scavenge reactive oxygen species (Parnham and Sies, 2000). Thus ebselen replaces endogenous glutathione peroxidase activity, which is inactivated by excess reactive oxygen species (Asahi et al., 1997; Pigeolet and Remacle, 1991).

Infarct volume measured 1 week after a 2 h ischemic insult was not modified by ebselen administration. It must be noted that this ischemic insult induced 30% mortality on average, with deaths always occurring within 12–48 h. Moreover, surviving rats suffered up to 25% body weight

loss and cerebral infarcts amounting to more than 25% of the ipsilateral hemisphere. Therefore, the neuroprotective efficacy of ebselen, even at high single doses, is limited by the severity of the ischemic insult, and is not maintained after a delayed endpoint for neuroprotection assessment. Our measurements of brain reduced glutathione showed that ebselen administration replaces ischemia-inactivated glutathione peroxidase activity, thus contributing to reduced glutathione consumption and at least partial reduction of oxidative stress. However, final brain damage after cerebral ischemia not only depends on direct macromolecular and cellular damage by reactive oxygen species. Specific signal transduction pathways, including genomic mechanisms, are activated by oxidative stress (Chan, 2001). Such signalling involves mitochondria, DNA repair enzymes and transcription factors. This mechanism, together with other delayed mechanisms such as iNOS induction and secondary inflammation could have escaped initial ebselen treatment and contributed to apoptosis/necrosis for delayed progression of brain infarct. In the light of the present results, administration of repeated ebselen doses along the reperfusion time, or the use of a combination therapy with ebselen and another antioxidant or non-antioxidant drug (Schmid-Elsaesser et al., 1999a,b) deserve further investigation. The ability of the new intravenous formulation of ebselen, alone (Imai et al., 2003) or in combination with thrombolytic therapy (Lapchak and Zivin, 2003), to produce a maintained neuroprotective effect after delayed evaluation should be studied in experimental ischemia and extended to clinical trials. This could help to improve the beneficial effects reported for ebselen in clinical trials (Ogawa et al., 1999; Yamaguchi et al., 1998), which are restricted to short-term administration and appear less consistent in middle cerebral artery occlusion patients when compared to patients suffering less severe acute ischemic strokes.

In conclusion, single-dose administration of ebselen does not reduce the size of brain infarcts resulting from severe focal cerebral ischemia in rats, despite modest preservation of intraischemic perfusion by high-dose ebselen. In contrast to previous studies with relatively earlier endpoints, we delayed the measurement of infarct volume to 1 week after the ischemic insult.

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References

Alegría, A., Barberá, R., Clemente, G., Farré, R., García, M.J., Lagarda, M.J., 1996. Selenium and glutathione peroxidase reference values in whole blood and plasma of a reference population living in Valencia, Spain. J. Trace Elem. Med. Biol. 10, 223–228.

Asahi, M., Fujii, J., Takao, T., Kuzuya, T., Hori, M., Shimonishi, Y., Taniguchi, N., 1997. The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor. J. Biol. Chem. 272, 19152–19157.

Bederson, J.B., Pitts, L.H., Germano, S.M., Nishimura, M.C., Davis, R.L., Bartkowski, H.M., 1986. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17, 1304–1308.

Chan, P.C., 2001. Reactive oxygen radicals in signaling and damage in the ischemic brain. J. Cereb. Blood Flow Metab. 21, 2–14.

Corbett, D., Nurse, S., 1998. The problem of assessing effective neuroprotection in experimental cerebral ischemia. Prog. Neurobiol. 54, 531–548.

Cuzzocrea, S., Riley, D.P., Caputi, A.P., Salvemini, D., 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol. Rev. 53, 135–159.

Dawson, D.A., Masayasu, H., Graham, D.I., Macrae, I.M., 1995. The neuroprotective efficacy of ebselen (a glutathione peroxidase mimic) on brain damage induced by transient focal cerebral ischemia in the rat. Neurosci. Lett. 185, 65–69.

Delanty, N., Dichter, M.A., 2000. Antioxidant therapy in neurological disease. Arch. Neurol. 57, 1265–1270.

Gilgun-Sherki, Y., Melamed, E., Offen, D., 2001. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40, 959–975.

Hoshida, S., Kuzuya, T., Nishida, M., Yamashita, M., Hori, M., Kamada, T., Tada, M., 1994. Ebselen protects against ischemia–reperfusion injury in a canine model of myocardial infarction. Am. J. Physiol. 267, H2342–H2347.

Imai, H., Masayasu, H., Dewar, D., Graham, D.I., Macrae, I.M., 2001. Ebselen protects both gray and white matter in a rodent model of focal cerebral ischemia. Stroke 32, 2149–2154.

Imai, H., Graham, D.I., Masayasu, H., Macrae, I.M., 2003. Antioxidant ebselen reduces oxidative damage in focal cerebral ischemia. Free Radic. Biol. Med. 34, 56–63.

Kagansky, N., Levy, S., Knobler, H., 2001. The role of hyperglycemia in acute stroke. Arch. Neurol. 58, 1209–1212.

Knollema, S., Elting, J.W., Dijkhuizen, R.M., Nicolay, K., Korf, J., Ter Horst, G.J., 1996. Ebselen (PZ-51) protects the caudate putamen against hypoxia/ischemia induced neuronal damage. Neurosci. Res. Commun. 19, 47–56.

Kondoh, S., Nagasawa, S., Kawanishi, M., Yamaguchi, K., Kajimoto, S., Ohta, T., 1999. Effects of ebselen on cerebral ischemia and reperfusion evaluated by microdialysis. Neurol. Res. 21, 682–686.

Kurihara, J., Katsura, K., Siesjo, B.K., Wieloch, T., 2004. Hyperglycemia and hypercapnia differently affect post-ischemic changes in protein kinases and protein phosphorylation in the rat cingulate cortex. Brain Res. 995, 218–225.

Lapchak, P.A., Zivin, J.A., 2003. Ebselen, a seleno-organic antioxidant, is neuroprotective after embolic strokes in rabbits. Synergism with low-dose tissue plasminogen activator. Stroke 34, 2013–2018.

Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20, 84–91.

Matés, J.M., 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153, 83–104.

Mori, T., Asano, T., Matsui, T., Muramatsu, H., Ueda, M., Kamiya, T., Katayama, Y., Abe, T., 1999. Intraluminal increase of superoxide anion following transient focal cerebral ischemia in rats. Brain Res. 816, 350–357.

Namura, S., Nagata, I., Takami, S., Masayasu, H., Kikuchi, H., 2001. Ebselen reduces cytochrome *c* release from mitochondria and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. Stroke 32, 1906–1911.

Ogawa, A., et al., for the Ebselen Study Group, 1999. Ebselen in acute middle cerebral artery occlusion: a placebo-controlled, double-blind clinical trial. Cerebrovasc. Dis. 9, 112–118.

Oliver, C.N., Starke-Reed, P.E., Stadtman, E.R., Liu, G.J., Carney, J.M., Floyd, R.A., 1990. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc. Natl. Acad. Sci. U. S. A. 87, 5144–5147.

Parnham, M., Sies, H., 2000. Ebselen: prospective therapy for cerebral ischemia. Expert Opin. Investig. Drugs 9, 607–619.

Peters, O., Back, T., Lindauer, U., Busch, C., Megow, D., Dreier, J., Dirnagl, U., 1998. Increased formation of reactive oxygen species after permanent and reversible middle cerebral artery occlusion in the rat. J. Cereb. Blood Flow Metab. 18, 196–205.

Pigeolet, E., Remacle, J., 1991. Susceptibility of glutathione peroxidase to proteolysis after oxidative alteration by peroxides and hydroxyl radicals. Free Radic. Biol. Med. 11, 191–195.

Polidori, M.C., Frei, B., Cherubini, A., Nelles, G., Rordorf, G., Keaney, J.F., Schwamm, L., Mecocci, P., Koroshetz, W.J., Beal, M.F., 1998. Increased plasma levels of lipid hydroperoxides in patients with ischemic stroke. Free Radic. Biol. Med. 25, 561–567.

Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W., Potter, D.W., 1980. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal. Biochem. 106, 55–62.

Salom, J.B., Ortí, M., Centeno, J.M., Torregrosa, G., Alborch, E., 2000. Reduction of infarct size by the NO donors sodium nitroprusside and spermine/NO after transient focal cerebral ischemia in rats. Brain Res. 865, 149–156.

Schmid-Elsaesser, R., Zausinger, S., Hungerhuber, E., Baethmann, A., Reulen, H.J., 1998. A critical reevaluation of the intraluminal thread model of focal cerebral ischemia. Evidence of inadvertent premature reperfusion and subarachnoid hemorrhage in rats by laser-doppler flowmetry. Stroke 29, 2162–2170.

Schmid-Elsaesser, R., Hungerhuber, E., Zausinger, S., Baethmann, A., Reulen, H.J., 1999a. Neuroprotective efficacy of combination therapy with two different antioxidants in rats subjected to transient focal ischemia. Brain Res. 816, 471–479.

Schmid-Elsaesser, R., Zausinger, S., Hungerhuber, E., Baethmann, A., Reulen, H.J., 1999b. Neuroprotective effects of combination therapy with tirilazad and magnesium in rats subjected to reversible focal cerebral ischemia. Neurosurgery 44, 163–171.

Schurr, A., 2002. Bench-to-bedside review: a possible resolution of the glucose paradox of cerebral ischemia. Crit. Care 6, 330–334.

Singhal, A.B., Wang, X., Sumii, T., Mori, T., Lo, E.H., 2002. Effects of normobaric hyperoxia in a rat model of focal cerebral ischemia–reperfusion. J. Cereb. Blood Flow Metab. 22, 861–868.

Takasago, T., Peters, E.E., Graham, D.I., Masayasu, H., Macrae, I.M., 1997. Neuroprotective efficacy of ebselen, an anti-oxidant with anti-inflammatory actions, in a rodent model of permanent middle cerebral occlusion. Br. J. Pharmacol. 122, 1251–1256.

Tombaugh, G.C., Sapolsky, R.M., 1993. Evolving concepts about the role of acidosis in ischemic neuropathology. J. Neurochem. 61, 793–803.

Wass, C.T., Lanier, W.L., 1996. Glucose modulation of ischemic brain injury: review and clinical recommendations. Mayo Clin. Proc. 71, 801–812.

Yamaguchi, T., et al., for the Ebselen Study Group, 1998. Ebselen in acute ischemic stroke. A placebo-controlled, double-blind clinical trial. Stroke 29, 12–17.