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EFECTO NEUROPROTECTOR DE LOS ANTIOXIDANTES EN UN MODELO DE ISQUEMIA CEREBRAL AGUDA

TESIS

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I. RESUMEN

El conocimiento sobre la fisiopatología de la enfermedad cerebral isquémica se deriva, esencialmente, de los estudios realizados en animales de laboratorio. Ahora se sabe que la isquemia cerebral aguda produce una serie de alteraciones bioquímicas muy compleja que evoluciona en el tiempo y en el espacio, y culmina con el daño funcional y la muerte celular. También se sabe que los radicales libres y el estrés oxidativo están estrechamente ligados en dicho proceso y que varios antioxidantes tienen efecto neuroprotector porque disminuyen el volumen del infarto en los modelos experimentales tradicionales de isquemia cerebral. Sin embargo, la información sobre su capacidad de reducir la mortalidad y mejorar el déficit neurológico derivados de la isquemia es muy limitada. En este trabajo se estudió el efecto de cuatro antioxidantes sobre la mortalidad y la discapacidad neurológica producidas en el ratón por el corte secuencial de las arterias carótidas comunes (SSACC). El estudio se llevó a cabo en ratones machos envejecidos (40-60 semanas de edad), a los cuales, bajo anestesia con éter, se les disecó la arteria carótida común izquierda y, excepto en el grupo "sham", se seccionó entre dos ligaduras. Treinta y dos días después, grupos de 18 a 22 animales se asignaron de manera aleatoria a diferentes tratamientos experimentales, antes de la anestesia y sección de la carótida común derecha. Quince min después, se administró por vía i.p. alguno de los siguientes fármacos: ácido ascórbico (AA, 500 mg/kg), ácido dihidrolipoico (DHLA, 100 mg/kg), *t*-butilhidroquinona (*t*-BHQ, 100 mg/kg), fenilbutilnitrona (PBN, 100 mg/kg) o solución salina. Los animales se evaluaron neurológicamente antes (basal) y 24, 48 y 72 horas después de la administración farmacológica. Con los datos del examen neurológico se aplicó una escala que determina el grado de discapacidad neurológica de cada animal; esta escala comprende 10 pasos progresivos, iniciando en 0 (normal) y extendiéndose hasta 10 (muerte debida a SSACC). En esta escala, el puntaje más alto refleja mayor daño funcional. Se encontró que AA y DHLA, pero no t-BHQ ni PBN, disminuyen la mortalidad y la discapacidad neurológica causados por la SSACC. Los índices de sobrevida a las 24, 48 y 72 horas fueron 86, 71 y 48% y 75, 65 y 65% para AA y DHLA, respectivamente, los cuales representan una prolongación significativa en comparación con los animales tratados con solución salina (60, 35 y 35%). Los puntajes de discapacidad neurológica a las 24, 48 y 72 horas fueron de 5.2, 6.2, y 7.5 y 4.6, 5.3, y 5.8 para AA y DHLA, respectivamente, los cuales fueron significativamente más bajos que los alcanzados en animales que recibieron solución salina (7.6, 8.6, y 8.6). Concluimos que, de los antioxidantes probados, el DHLA es el más eficaz para disminuir la mortalidad y el déficit neurológico producido por la SSACC; asimismo, que nuestra estrategia para evaluar la eficacia neuroprotectora es suficientemente sensible para confirmar la validez y utilidad de nuestro modelo experimental de isquemia cerebral.

II.   INTRODUCCION

2.1 Isquemia cerebral aguda

La isquemia cerebral aguda es la consecuencia de una reducción severa del flujo sanguíneo, habitualmente debida a la oclusión de una arteria principal por un trombo o un émbolo. Si este evento se prolonga por más de 5 min, disminuye el aporte de sustratos, particularmente de oxígeno y glucosa, se alteran los procesos energéticos que mantienen los gradientes iónicos y los potenciales eléctricos de membrana. Esta crisis dispara varios procesos a nivel celular y subcelular y se inician los eventos que constituyen la cascada isquémica (Hossmann, 1994) y que culminan con alteraciones funcionales y la muerte celular. Este padecimiento, denominado enfermedad vascular cerebral (EVC), es la causa principal de discapacidad física y mental en los adultos, y se mantiene como la tercer causa más frecuente de muerte en la población mayor de 45 años (American Stroke Association, 2003). Debido a que la incidencia de esta enfermedad aumenta con la edad (Wardlaw y cols., 1996), se estima que el número de pacientes será mayor en las próximas décadas, conforme avanza el envejecimiento de la población.

Los estudios experimentales han revelado la presencia de algunas regiones, dentro del territorio cerebral isquémico, donde se mantiene parcialmente el flujo sanguíneo; a dichas regiones se les denomina zonas de penumbra isquémica y constituyen áreas potencialmente rescatables (Astrup y cols., 1981; Hakim, 1987; Baron, 1999; Heiss y cols., 2001; Fisher, 2004). Las neuronas de esas zonas mantienen parcialmente el suministro de energía, la homeostasis iónica y la integridad de sus membranas (Astrup y cols., 1981), siendo eléctrica y funcionalmente silentes, pero viables durante un periodo limitado de tiempo. Se estima que alrededor del 50% del volumen de la penumbra progresa a infarto (Belayev y cols., 1997) y que la sobrevida celular y la recuperación funcional son proporcionales al volumen que escapa de la muerte, constituyendo el blanco primario de la protección farmacológica (Dirnagl y cols., 1999; Baron, 1999; Ginsberg, 2003).

Las manifestaciones clínicas de la enfermedad vascular cerebral aguda dependen de la localización, severidad, y duración del déficit de perfusión. En general, se presenta una gran variedad de alteraciones sensoriales y motoras, incluyendo temblor, pérdida de la coordinación muscular, parálisis parciales (Baumlin y Richardson, 1997; Caplan y Hon, 2004). También se puede presentar disfunción cortical superior, que se manifiesta como amnesia, demencia y delirio, además de trastornos del lenguaje (Patel y cols., 2003). Un 25% de los pacientes con EVC aguda muere en el primer mes, y un 50% de los sobrevivientes quedan física y

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mentalmente discapacitados (American Stroke Association, 2003).

2.2 Cascada bioquímica de daño isquémico

a. Crisis energética. Al limitar el aporte de oxígeno y de glucosa, la isquemia cerebral aguda altera los procesos energéticos que mantienen la transmisión sináptica, el transporte de sodio y potasio, y la integridad estructural de la célula (Hossmann, 1987; Back, 1998). En un principio, la cascada bioquímica de daño sigue una secuencia determinada. En pocos segundos, como una respuesta para ahorrar energía, el tejido cerebral afectado detiene su actividad eléctrica. La evidencia disponible señala que este evento es inducido por la salida del K^+ intracelular (Martin y cols., 1994; Lee y cols., 2000) y resulta una hiperpolarización transitoria de la membrana plasmática. A pesar de esto, el ATP se consume gradualmente debido a la inhibición de la fosforilación oxidativa y al gasto de energía que implica mantener el transporte iónico de Na^+ y K^+ y la integridad estructural (Hossmann, 1987; Ames, 1992). De manera que, después de 5 min de isquemia, el ATP cae hasta en un 90%; a estos niveles, falla la actividad de la Na^+-K^+-ATPasa en la membrana plasmática y en el retículo endoplásmico. Esta crisis dispara la entrada celular del Na^+ y la salida concomitante del K^+, asociado con una despolarización severa que, en suma, causa un fenómeno macroscópico conocido como despolarización anóxica (Sun y Foudin, 1984). Como consecuencia de esta despolarización prolongada, se abren los canales presinápticos para el Ca^{2+} sensibles a voltaje y aumenta el Ca^{2+} intracelular, produciéndose una liberación excesiva de neurotransmisores, especialmente de glutamato.

Cabe mencionar que los cambios en la relación Na^+/K^+ y en los niveles de ATP son mucho menos importantes en las áreas de penumbra; en estas zonas, la despolarización anóxica es reemplazada por despolarizaciones intraisquémicas episódicas o intermitentes (Takeda y cols., 1993), caracterizadas por una duración menor y una repolarización rápida de la célula. Sin embargo, con el tiempo, el ATP disminuye severamente y se produce despolarización irreversible (Ginsberg, 2003).

b. Excitotoxicidad. Los cambios masivos en los gradientes iónicos ya mencionados producen la liberación excesiva de neurotransmisores, especialmente aminoácidos excitadores (glutamato). Los estudios en los que se han determinado niveles extracelulares de glutamato reportan concentraciones entre 50 y 80 μM durante varias horas en el centro isquémico focal (Shimada y cols., 1989; Baker y cols., 1995), y de 200 μM en el estriado después de 2 h de isquemia global (Obrenovitch y cols., 1993). También se ha reportado que la fuente principal

de este glutamato son las terminales presinápticas y somatodendríticas (Mitani y cols., 1994; Dirnagl y cols., 1999; Rossi y cols., 2002), además de la falla en los procesos para su recaptura, posiblemente vía de una reversión del transportador de Na^+-glutamato (Dirnagl y cols., 1999).

Las cantidades excesivas de glutamato sobreestimulan a los receptores ionotrópicos, los cuales se agrupan en tres familias: (i) receptores AMPA, mediadores de la neurotransmisión rápida y que permiten la entrada de Na^+ y algo de Ca^{2+}; (ii) receptores kainato, que permiten la entrada de Na^+ y Ca^{2+}; y (iii) receptores N-metil-D-aspartato (NMDA) (Seeburg, 1993), vía principal para la entrada de Ca^{2+} a las neuronas. El receptor NMDA tiene un bloqueo por la molécula de Mg^{2+}. La activación de los receptores AMPA contribuye a la despolarización al quitar el bloqueo de la molécula de Mg^{2+} en el canal receptor NMDA que, consecuentemente, se abre y permite la entrada de Ca^{2+}. Estos procesos dan como resultado la entrada masiva de Ca^{2+} y Na^+; lo cual determina la entrada pasiva de agua produciendo edema celular y tisular. La entrada de Ca^{2+} causa la liberación de las reservas del retículo endoplásmico, por la vía de un mecanismo dependiente de Ca^{2+} (Mody y MacDonald, 1995). La disipación de los gradientes de Na^+ revierte la función del intercambiador Na^+/ Ca^{2+} y permite la entrada ulterior de más Ca^{2+} a la célula (Choi, 1988; Lobner y Lipton, 1993).

El glutamato también actúa sobre los receptores metabotrópicos que, por la vía de la activación de fosfolipasa C, genera inositol trifosfato (IP3). El IP3 se une a su receptor sobre el retículo endoplásmico y se libera Ca^{2+}, aumentando la concentración de calcio intracelular. La activación de fosfolipasa A2 por glutamato libera al ácido araquidónico, el cual también puede liberar Ca^{2+} del retículo endoplásmico (Farooqui y Horrocks, 1994). Adicionalmente, la disminución del ATP debilita los mecanismos encargados de remover el Ca^{2+} citoplásmico en la mitocondria, retículo endoplásmico y membrana plasmática. Se ha reportado que el Ca^{2+} intracelular se eleva un 25% después de la isquemia global (Kass y Lipton, 1986) y en el centro isquémico focal (Harris y cols., 1981). Los incrementos son mucho menores en la zona de penumbra, y se han relacionado a los periodos de despolarización intraisquémica (Kristian y cols., 1998).

La elevación sostenida de Ca^{2+} intracelular dispara una serie de procesos dependientes de Ca^{2+} que finalmente conducen a la muerte celular. Dichos procesos incluyen: activación persistente de la proteína cinasa C (PKC), ruptura de proteínas estructurales, fosforilación sostenida de proteínas, y activación de proteasas, tales como calpaínas y endonucleasas. Una de las vías citotóxicas del Ca^{2+} lleva a la generación de especies reactivas de oxígeno y

nitrógeno. A través de la activación de sintasas del óxido nítrico (ON$^\bullet$), constitutivas (SON endotelial y SON neuronal) e inducible (iNOS), se forman cantidades excesivas de ON$^\bullet$. También se activan fuentes de superóxido a través de las enzimas xantina oxidasa y fosfolipasa A$_2$. Además, el exceso de Ca^{2+} en la mitocondria desacopla la fosforilación oxidativa, lo que disminuye el suministro de energía y aumenta la producción de radicales libres, los cuales alteran la permeabilidad mitocondrial al formar poros de transición (Neumar, 2000).

El daño al ADN, vía endonucleasas y radicales libres, dispara procesos autodestructivos muy complejos que involucran la expresión de genes. La evidencia de apoptosis neuronal postisquémica incluye (1) fragmentación característica de ADN; (2) regulación a la alza de factores proapoptóticos (p53, Fas, ligando Fas, FNT-α, receptor-FNT, Bcl-Xs, Bax); y (3) activación de caspasas (Neumar, 2000). Existe evidencia de que la mitocondria es una estructura clave para la inducción de esta muerte celular programada. Se ha observado que reducciones moderadas en la producción de ATP mitocondrial pueden disparar mecanismos apoptóticos, y los estudios más recientes señalan a la liberación de caspasa 9 (Krajewski y cols., 1999), citocromo c, y del factor inductor de apoptosis, a partir de la mitocondria, como iniciadores de la muerte celular apoptótica.

c. Inflamación. La inflamación es otro componente que contribuye al daño celular después de la isquemia cerebral (Danton y Dietrich, 2003). Los receptores de adhesión endotelial son regulados a la alza y los leucocitos se adhieren a las paredes de los vasos sanguíneos, invaden el parénquima y liberan citosinas, tales como el factor de necrosis tumoral α, interleucina 1 e interleucina 6 (Barone y Feuerstein, 1999).

Estos mecanismos de daño neuronal pueden ocurrir en secuencia o en paralelo y, en todos los casos, se ha encontrado que se entrecruzan. El curso que sigan y la contribución de cada mecanismo puede variar dependiendo de factores como la edad y la severidad del daño (Neumar, 2000). En resumen, el daño cerebral isquémico es multidimensional en origen y ofrece un rango muy amplio de sitios para la intervención neuroprotectora.

Conviene hacer notar que esta introducción trata con más detalle la fisiopatología de la isquemia cerebral aguda estrechamente relacionada con el estrés oxidativo y refiere los sitios donde los compuestos antioxidantes pueden interrumpir los eventos que conducen a la lesión y muerte celular.

2.3 Estrés oxidativo en la cascada isquémica

a. Radicales libres. Desde el punto de vista químico un radical libre es cualquier compuesto que contenga uno o más electrones no pareados en su orbital externo (Halliwell y Gutteridge, 1999). Los electrones impares de una molécula alteran su reactividad, usualmente haciéndola más reactiva, porque actúan como aceptores de electrones y abstraen los electrones de otras moléculas. A la pérdida de este electrón, por lípidos, proteínas, ADN, y otras biomoléculas, se le llama oxidación y a los radicales libres se les conoce como agentes oxidantes. Las moléculas oxidadas, al quedar con un electrón no pareado, se convierten en otro radical y son capaces de continuar las reacciones indefinidamente hasta reaccionar con moléculas antioxidantes o destruir las estructuras celulares.

En las células aeróbicas, las reacciones de los radicales libres más importantes involucran al oxígeno molecular (O_2) y a sus especies reactivas: superóxido ($O_2^{\bullet-}$), peróxido de hidrógeno (H_2O_2) y al radical hidroxilo ($^{\bullet}OH$); al óxido nítrico ($ON^{\bullet}$) y sus derivados: peroxinitrito ($ONOO^-$); ácido nitroso ($ONOOH$), y al dióxido de nitrógeno ($^{\bullet}NO_2$); a los peróxidos orgánicos incluyendo al hidroperóxido ($LOOH$); y a los metales de transición, entre ellos al hierro (Fe^{2+}) y al cobre (Cu^{2+}) (Halliwell y Gutteridge, 1999). La mayoría de las especies reactivas de oxígeno se forman por la reducción (ganancia de electrones) incompleta del O_2. La adición secuencial de un electrón (e^-) al oxígeno molecular (O_2), produce la formación del radical anión superóxido, del peróxido de hidrógeno, y la formación del radical hidroxilo (Werns y Lucchesi, 1990):

$$O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH \qquad \text{(ec. 1)}$$
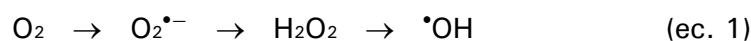
b. Fuentes de radicales libres en la isquemia cerebral. Durante la isquemia cerebral se crean una serie de interacciones entre varias vías metabólicas y catabólicas que generan radicales libres, algunas de ellas se desencadenan al ocurrir la reperfusión (Fig. 1) (Traystman y cols., 1991; Phillis, 1994; Hall, 1997). Cabe mencionar que en la zona de penumbra, donde el flujo sanguíneo es parcial, se presentan los mismos procesos que desencadena la reperfusión.

i. Fuga de electrones en mitocondria. En condiciones normales, el O_2 se reduce a H_2O (adición secuencial de cuatro electrones:) por la cadena transportadora de electrones *(e^-)*, sin la producción de radicales de oxígeno (Traystman y cols., 1991; Turrens, 1997):

$$4H^+ + 4e^- + O_2 \rightarrow 2H_2O \qquad \text{(ec. 2)}$$
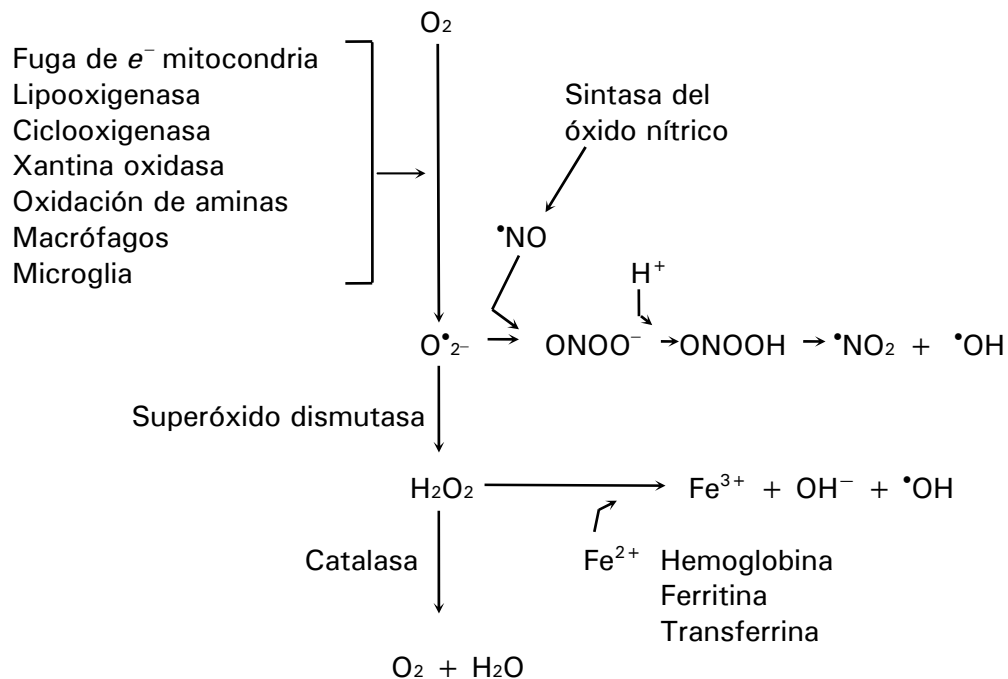
Figura 1. Fuentes potenciales de especies reactivas de oxígeno y nitrógeno durante la isquemia y reperfusión cerebral (tomado de Hall, 1997).

La presencia de oxígeno al final de la cadena respiratoria favorece el mantenimiento de los miembros del sistema transportador en un estado oxidado (existe transferencia pero no sobran ni se fugan electrones). En contraste, durante la isquemia, cuando el suministro de oxígeno es limitado, la cadena transportadora de electrones llega a ser altamente reductora (donadora de e^-); si en estas condiciones ocurre reoxigenación como en la recanalización del vaso sanguíneo, la producción de superóxido ($O_2^{\bullet-}$) aumenta al potenciarse por las condiciones reducidas presentes durante la isquemia (Phillis, 1994). Se ha propuesto a la región ubiquinona (QH2)–citocromo b (Cit b) como el sitio más importante en la producción de radicales superóxido ($O_2^{\bullet-}$) cuando la mitocondria está en un estado reducido máximo (Cino y Del Maestro, 1989; Turrens, 1997):

$$QH_2 + e^- \text{ (Cit } b \text{ o } c) \rightarrow {}^\bullet QH^- + O_2 \rightarrow Q + O_2^{\bullet-} \qquad \text{(ec. 3)}$$
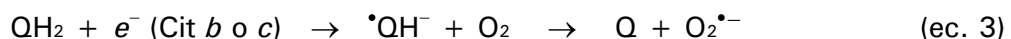
ii. Metabolismo de los ácidos grasos. Se ha reportado que durante la isquemia se incrementa la liberación de los ácidos grasos, en especial los ácidos grasos poliinsaturados, como el ácido araquidónico (Rao y cols., 1999; Yang y cols., 1999), como consecuencia de la actividad de fosfolipasa C (activada por despolarización) y fosfolipasa A2 (cPLA2) (activada por el incremento de Ca_i^{2+}) (Yang y cols., 1999), además de la falla en la síntesis de fosfolípidos como consecuencia de la disminución del ATP. La severidad del daño isquémico

correlaciona con el nivel de acumulación de ácidos grasos (Abe y cols., 1987) y se sabe que el incremento es mayor en las regiones más susceptibles al daño isquémico (Westerberg y cols., 1987; Phillis, 1994; Yang y cols., 1999). En estas condiciones, el ácido araquidónico rápidamente se metaboliza por las enzimas lipooxigenasa (LOX) y ciclooxigenasa (COX) (Phillis y O'Regan, 2003). Al adicionar dos moléculas de O_2 a un ácido graso insaturado, la COX produce prostaglandina G_2, la cual es rápidamente peroxidada a prostaglandina H_2 (PGH_2) con la producción concomitante de $O_2^{\bullet-}$ (Armstead y cols., 1988; Krause y cols., 1988). Las conversiones subsecuentes de PGH_2 producen prostaglandinas, prostaciclinas y tromboxanos (Gaudet y cols., 1980; Warner y cols., 2004), moléculas vasoactivas que pueden agravar el daño isquémico. Los radicales superóxido también pueden generarse por la vía de la lipooxigenasa que produce leucotrienos (Phillis, 1994).

iii. Metabolismo de purinas. En condiciones normales, la hipoxantina es convertida en xantina por la enzima xantina deshidrogenasa (XDH). Sin embargo, durante la isquemia, a través de un proceso Ca^{2+}-dependiente, las proteasas convierten a la XDH en xantina oxidasa. Además, que durante la isquemia el ATP se utiliza pero no se resintetiza, y sus metabolitos como AMP, adenosina, inosina, hipoxantina y xantina se acumulan (Morimoto y cols., 1982). La xantina oxidasa utiliza al O_2 como su aceptor de electrones y cataliza la producción de $O_2^{\bullet-}$ (Phillis, 1994).

iv. Oxido nítrico y sus derivados. El incremento en la producción de $ON^{\bullet}$ durante la isquemia y la reperfusión (Wei y cols., 1999), en un pricipio, se debe a las formas constitutivas de la sintasa del óxido nítrico (NOS, por sus siglas en inglés) (NOS neuronal y NOS endotelial), las cuales son activadas por el incremento citosólico de Ca^{2+} (Malinski y cols., 1993). El complejo Ca^{2+}/calmodulina aumenta la actividad de la enzima, la cual cataliza la formación de $ON^{\bullet}$ a partir de la L–arginina (Phillis, 1994).

La interacción entre las especies reactivas con otros componentes tisulares produce diversos radicales. De gran importancia es la interacción del $O_2^{\bullet-}$ con el $ON^{\bullet}$, la cual genera el anión peroxinitrito ($ONOO^-$) (Eliasson y cols., 1999), oxidante fuerte de grupos sulfhidrilos que, a un pH fisiológico, sufre protonación formando ácido peroxinitroso (ONOOH), molécula química y termodinámicamente muy inestable que espontáneamente se descompone para producir a los radicales hidroxilo ($^{\bullet}OH$) y dióxido de nitrógeno ($^{\bullet}NO_2$), y una fuente de $^{\bullet}OH$ independiente de la catálisis por hierro (Fig. 1):

$$O_2^{\bullet-} + ON^{\bullet} \rightarrow ONOO^- + H^+ \rightarrow ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \qquad \text{(ec. 4)}$$
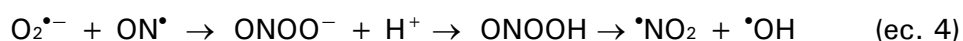
Así el $^\bullet NO_2$ puede iniciar la peroxidación de lípidos o nitrar e inactivar proteínas celulares sobre los residuos de tirosina (Espey y cols., 2000). Un aspecto importante de este mecanismo es que el ácido peroxinitroso (ONOOH), en comparación con otros radicales libres, tiene una vida media prolongada, siendo potencialmente más difundible. De esta manera, puede ofrecer un mecanismo de daño en sitios remotos (Hall, 1997). Esta fuente de daño por radicales involucra células endoteliales, neutrófilos, macrófagos y microglías, las cuales pueden producir $ON^\bullet$ concomitantemente con $O_2^{\bullet-}$ (Hall, 1997; Nakazawa y cols., 1996).

El grado de producción de radicales libres parece ser proporcional a la duración y severidad de la isquemia, y a la posibilidad de que exista o no reperfusión (reoxigenación) (Sakamoto y cols., 1991). Se ha reportado que en el centro isquémico de la región cortical la producción de radicales disminuye o permanece durante la isquemia, mientras que se incrementa durante la reperfusión (Kim y cols., 2002). En la zona de penumbra, donde parcialmente se mantiene la concentración de O_2, la producción es elevada tanto en la isquemia como durante la reperfusión.

De gran importancia en este proceso es la liberación de hierro (Fe^{2+}), catalizador esencial en la formación del radical $^\bullet OH$ (altamente reactivo y citotóxico) a partir de H_2O_2 (reacción de Fenton:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^\bullet OH \qquad \text{(ec. 5)}$$

Además, el hierro también participa en la formación de los radicales orgánicos alcoxilo ($LO^\bullet$) y peroxilo ($LOO^\bullet$), a través de una lipoperoxidación Hierro-dependiente (Krinsky, 1992; Hall, 1997):

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + OH^- + LO^\bullet \qquad \text{(ec. 6)}$$

$$LOOH + Fe^{3+} \rightarrow Fe^{2+} + OH^+ + LOO^\bullet \qquad \text{(ec. 7)}$$
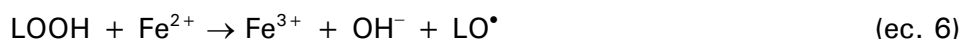
Por otro lado, el cerebro cuenta con varias defensas contra las especies reactivas, incluyendo ácido ascórbico, α-tocoferol, el tripéptido endógeno glutation, y enzimas antioxidantes como superóxido dismutasa, glutation peroxidasa y catalasa. Sin embargo, aún cuando la expresión de enzimas antioxidantes aumenta en respuesta a la isquemia y reperfusión (Fukui y cols., 2002; Dirnagl y cols., 2003), la producción excesiva de oxidantes rebasa la capacidad antioxidante endógena y se produce daño conocido como estrés oxidativo. El exceso de radicales libres altera la función celular principalmente por dañar moléculas esenciales, como lípidos (membranas), carbohidratos, aminoácidos y proteínas, además de daño al ADN y ARN, conduciendo eventualmente a la muerte celular (Maxwell,

1995). Al proceso de estrés oxidativo se le considera como un mecanismo que precipita cambios patológicos en el tejido nervioso durante la isquemia y reperfusión cerebral (Hall y Braughler, 1989; Watson, 1993; Hall, 1997; Schaller, 2005).

c. Peroxidación de lípidos. La interacción de los radicales oxidantes con los fosfolípidos de las membranas produce lipoperoxidación. Este parece ser el mecanismo principal por el que los radicales libres dañan al tejido cerebral (Martinez-Vila e Irimia, 2001). El daño inicia cuando un radical altamente reactivo, como el radical $^\bullet OH$, remueve un átomo de hidrógeno de un ácido graso poliinsaturado de alguna membrana, dejando un electrón no pareado sobre un carbono (radical carbono o radical alquilo) (Fig. 2). Este evento es seguido por un rearreglo molecular que forma un dieno conjugado, el cual se combina con el oxígeno (su presencia propaga estas reacciones) creando al radical peroxilo (LOO$^\bullet$), y continúa la cascada de reacciones redox con los ácidos grasos adyacentes, generando radicales alcoxilo (LO$^\bullet$), los cuales tienen la capacidad de continuar la cascada de reacciones a la vez que libera hidroperóxidos lipídicos (LOOH). De esta manera, un radical puede convertir múltiples cadenas de ácidos grasos en hidroperóxidos lipídicos (Halliwell, 1994). La acumulación de LOOH en la membrana altera su función y puede causar su colapso. Además, los hidroperóxidos lipídicos pueden descomponerse y formar diversos productos altamente citotóxicos, incluyendo malonaldehído (MDA), 4-hidroxinonenal (HNE) y alcanos (Morrow y cols., 1990; Montuschi y cols., 2004).

Una vez iniciada esta secuencia de reacciones (Fig. 2), el hierro liberado durante la isquemia interviene activamente en la formación de más radicales orgánicos incluyendo LO$^\bullet$ y LOO$^\bullet$ a partir de LOOH (ec. 6 y ec. 7).

Se sabe que el hierro estimula la lipoperoxidación aún en ausencia del radical iniciador (Hall, 1997). Además, el tejido nervioso brinda un ambiente especialmente propicio para estas reacciones por su alto contenido de hierro, distribuido de manera paralela con la susceptibilidad regional a la lipoperoxidación (Zaleska y Floyd, 1985) y a la alta proporción de ácidos grasos poliinsaturados peroxidables, tales como el ácido linoleico y el ácido araquidónico (Hall, 1997).

En conjunto, la reacción en cadena iniciada por un radical oxida múltiples ácidos grasos, formando poros y dañando el soporte de proteínas de la membrana, como receptores, canales iónicos y enzimas; eventualmente, rompe la membrana celular (Siesjo y cols., 1989; Halliwell, 1994).
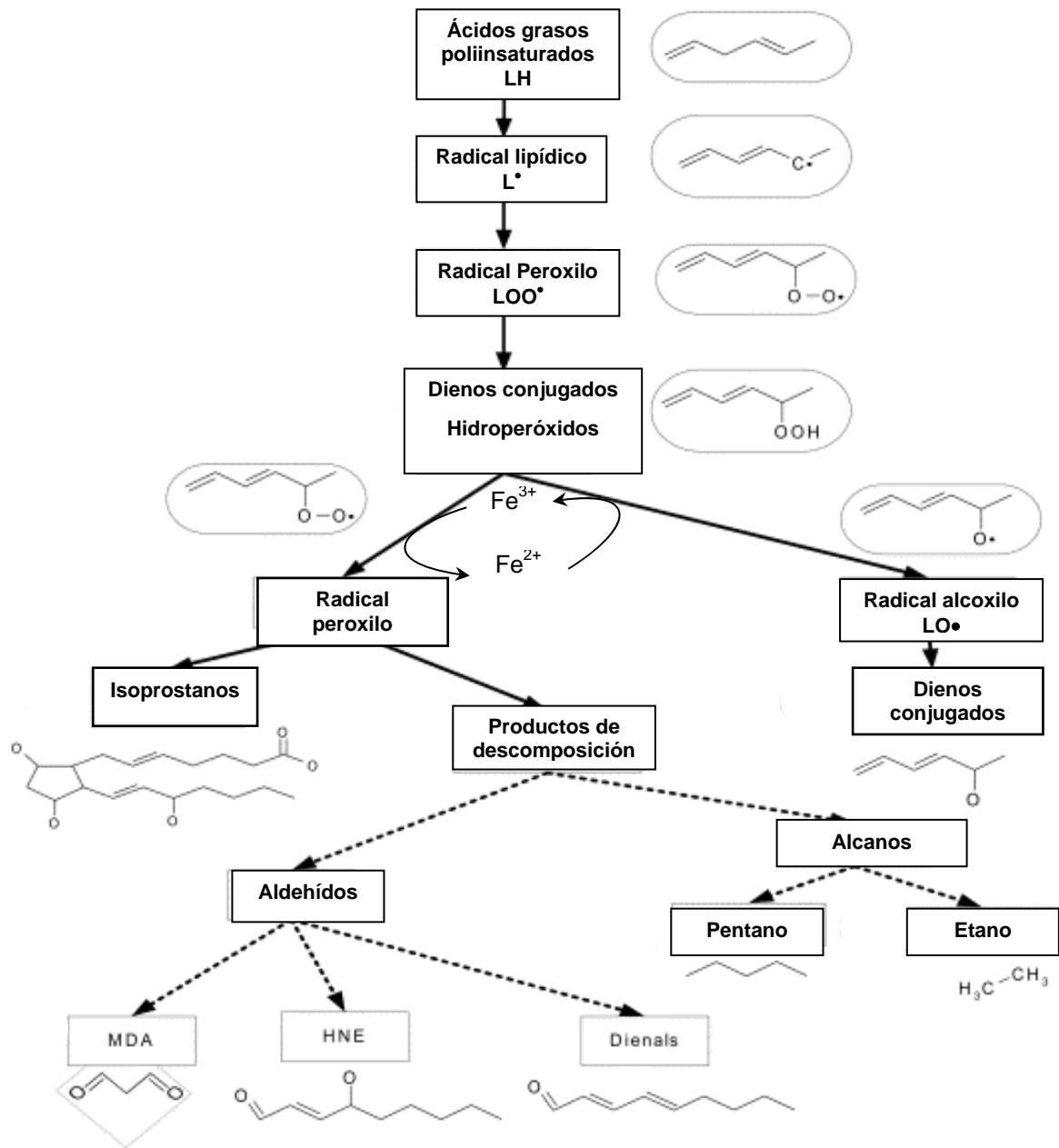
Figura 2. Vías y productos relacionados a la lipoperoxidación (tomado de Dotan y cols., 2004)

d. Daño a las proteínas. La oxidación de proteínas produce modificación de su carga, formación de puentes disulfuro del grupo tiol (-SH) y formación de enlaces covalentes y/o hidrofóbicos. Estas reacciones suelen causar pérdida de tioles libres, descarboxilación y desaminación, y la generación de grupos carbonilos. Las proteínas modificadas por la vía oxidativa activan a enzimas proteolíticas y son más susceptibles a la proteólisis (Nakazawa y cols., 1996).

e. Daño a los ácidos nucleicos. La oxidación de los ácidos ribo y deoxirribonucleicos

produce rompimiento de las cadenas de ADN, intercambio de cromatina, entrecruzamientos ADN-ADN y proteína-ADN, además de modificación de las bases (Nakazawa y cols., 1996).

2.3.1 Sitios y mecanismos de acción de los antioxidantes

En apoyo de la participación importante de las especies reactivas de oxígeno y nitrógeno en el daño celular por isquemia y reperfusión cerebral, se tiene la evidencia de que un gran número de antioxidantes, con diferentes estructuras químicas y mecanismos de acción, son capaces de reducir el volumen de la lesión en modelos tradicionales de isquemia cerebral global y/o focal (Hall, 1997; Gilgun-Sherki y cols., 2002; Warner y cols., 2004).

Los antioxidantes son compuestos exógenos (naturales o sintéticos) y endógenos que actuando por mecanismos diversos inhiben el proceso de oxidación. Pueden actuar donando electrones, eliminando a la especie oxidante o a sus precursores, inhibiendo la formación de las especies reactivas, uniéndose a los metales iónicos que son catalizadores en la generación de especies reactivas, o interfiriendo con las fases de la lipoperoxidación (Gilgun-Sherki y cols., 2002).

Rice-Evans (1999) considera que los sistemas de defensa contra el daño por especies reactivas se pueden clasificar en tres grupos:

- Antioxidantes preventivos que suprimen la formación de radicales libres.
- Antioxidantes que inhiben la iniciación de las reacciones en cadena e interceptan la propagación al atrapar radicales libres.
- Antioxidantes involucrados en los procesos de reparación.

El requisito característico de las moléculas antioxidantes eficaces incluye un número de rasgos estructurales:

1. La presencia de grupos donadores de electrones o hidrógenos con apropiados potenciales de reducción en relación a las parejas redox de los radicales a ser atrapados (Bors y cols., 1990, 1995; Jovanovic y cols., 1994, 1995; Steenken y Neta, 1992).

2. La habilidad de deslocalizar al radical resultante (Bors y cols., 1990) en un radical fenoxilo, tal como el que deriva de α-tocoferol, en un radical ariloxilo como el derivado de los flavonoides, en un radical de la cadena de hidrocarbono poliinsaturado tal como el de β-caroteno, o en un radical tiilo tal como el ácido dihidrolipoico.

3. El potencial de quelar metales de transición (Thompson y cols., 1976; Morel y cols., 1993; Afanas'ev y cols., 1989; Paganga y cols., 1996) dependiente de la naturaleza

de los grupos funcionales y de su arreglo con las moléculas.

También de la interacción de los radicales antioxidantes con otras moléculas antioxidantes, reciclando al antioxidante original, así evita la disminución de ellos (Rice-Evans, 1999).

a. Efecto de los antioxidantes en los modelos de isquemia cerebral

i. Quelantes de metales. Como ya se comentó, durante y después de la isquemia cerebral, las reacciones de oxidación hierro-dependientes parecen contribuir de manera importante al daño tisular. Se han evaluado varios quelantes sintéticos, cuya acción primaria es disminuir la concentración de hierro o cobre libres. A través de este mecanismo, estos quelantes pueden inhibir la formación de radicales libres en dos niveles: sobre la reacción de Fenton (ec. 5) y sobre la lipoperoxidación hierro-dependiente (ec. 6 y 7) (Maxwell, 1995; Krinsky, 1992). Uno de estos quelantes, la deferoxamina, administrada antes o un poco después del episodio isquémico aumenta la sobrevida y mejora los parámetros funcionales en ratas (Palmer y cols., 1994), perros (Hurn y cols., 1995); y ratones (Sarco y cols., 2000). Sin embargo, no se observó ningún beneficio en isquemia cerebral completa en perros (Fleischer y cols., 1987), ni en ratones sometidos a sección secuencial de las arterias carótidas comunes (Rodriguez y cols., 2003b). El dexrazoxano, un compuesto quelante que *in vivo* se hidroliza para producir un quelante estructuralmente similar al EDTA, disminuye la cardiotoxicidad en pacientes con cáncer de mama tratados con antraciclinas (Speyer y cols., 1988), disminuye la acción diabetogénica del aloxano en ratones (El-Hage y cols., 1981), y aumenta significativamente la sobrevida y disminuye el déficit neurológico en ratones con isquemia cerebral severa (Rodriguez y cols., 2000b; Rodriguez y cols., 2003b).

ii. Inhibidores de xantina oxidasa. El alopurinol es un fármaco inhibidor de xantina oxidasa. Su administración disminuye las concentraciones de ácido úrico, de xantina, y de dienos conjugados (Marro y cols., 1994; Nihei y cols., 1989), mantiene el ATP (Williams y cols., 1992), y reduce el edema (Patt y cols., 1988). Se ha reportado que este fármaco reduce significativamente el volumen del infarto (Martz y cols., 1989) en ratas con oclusión de la arteria cerebral media.

iii. Sobreexpresión de la sintasa del óxido nítrico. Se ha informado que la sobreexpresión de la sintasa del óxido nítrico endotelial por el tratamiento con inhibidores de la 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA) reductasa, como simvastatina, aumenta el flujo sanguíneo intraisquémico y reduce el volumen del infarto (Endres y cols., 1998; Amin-Hanjani

y cols., 2001).

iv. Inhibidores de la poli-ADP-ribosa polimerasa, (PARP). La PARP se activa en respuesta al daño a ADN. Se trata de un mecanismo reparador, pero también causa depleción de NAD y de ATP, por lo que potencialmente puede exacerbar el daño isquémico. Se ha demostrado que varios antagonistas de PARP tienen efecto protector en varios modelos de isquemia experimental (Abdelkarim y cols., 2001; Plaschke y cols., 2000), uno de los cuales observó protección más allá de 30 días posteriores a la isquemia (Ding y cols., 2001).

v. Miméticos del glutation. El glutation (GSH) es un tripéptido (γ-L-glutamil-L-cisteinilglicina) que en células de mamífero participa en muchas funciones fisiológicas, incluyendo la defensa contra los radicales oxidantes (Lu, 1999). Es el reductor para la glutation peroxidasa. La oxidación de sus grupos sulfhidrilo en el aminoácido cisteína une a dos moléculas de glutation (GSH) a través de un puente disulfuro para formar disulfuro de glutation (GSSG). La glutation reductasa NADH-dependiente cataliza la recuperación del glutation. Normalmente, el cerebro mantiene una alta relación de GSH/GSSG para su defensa antioxidante. La depleción total del glutation y una disminución en la relación GSH/GSSG son marcadores de estrés oxidativo en la isquemia cerebral y se podrían requerir hasta 72 horas para restablecer las concentraciones a valores normales (Namba y cols., 2001; Park y cols., 2000). Se ha demostrado empeoramiento del daño isquémico por la disminución farmacológica de glutation (Vanella y cols., 1993), y protección por la administración de YM737 (mimético del glutation) (Gotoh y cols., 1994), o N-acetilcisteína, un precursor del glutation.

vi. Inductores de enzimas antioxidantes. Se ha demostrado que la sobreexpresión de genes antioxidantes, como el de la glutation peroxidasa, protege a las neuronas contra la isquemia cerebral aguda; asimismo, que aumenta el volumen del infarto en ratones que carecen de este gen (Hoehn y cols., 2003). La sobreexpresión de los antioxidantes se puede adquirir por la terapia génica (Raymon y cols., 1997) o por factores de crecimiento (Spina y cols., 1992; Colton y cols., 1995). Una estrategia alternativa es el tratamiento con inductores de enzimas antioxidantes (Murphy y cols., 1991); estos inductores son oxidantes de baja potencia que incrementan la expresión de genes antioxidantes (Daniel, 1993). Existen reportes que la t-BHQ aumenta la expresión de γ-glutamilcisteína sintetasa y de glutation sintetasa (Huang y cols., 2000), ambas enzimas implicadas en la síntesis de glutation. Además, otros autores han reportado que t-BHQ aumenta la actividad de factores de

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transcripción, tales como AP-1 y NFκB, y del elemento de respuesta antioxidante (ARE, por sus siglas en inglés) (Pinkus y cols., 1996; Kang y cols., 2001; Yang y cols., 2002).

vii. Ácido ascórbico (vitamina C). El ácido ascórbico es una molécula antioxidante que es hidrosoluble y existe en altas concentraciones en el sistema nervioso central. El ácido ascórbico no cruza rápido ni fácilmente la barrera hematoencefálica. Se sabe que entra al líquido cefalorraquídeo por medio de un transporte activo a través del plexo coroideo (Rice, 2000). Su metabolito oxidado, el ácido dehidroascórbico, rápidamente atraviesa la barrera hematoencefálica y es retenido en su forma oxidada, ácido ascórbico (Agus y cols., 1997), aunque este parece ser un mecanismo menor. Sin embargo, la concentración en cerebro, predominantemente intracelular, es 10 veces mayor que en sangre (Frei y England, 1989; Schreiber y Trojan, 1991; Rose y Bote, 1993). El ácido ascórbico actúa como atrapador de radicales libres debido a su propiedad de donar electrones (Padh, 1990; Rice, 2000). Se trata de un atrapador de radicales libres, de amplio espectro y eficaz contra radicales peroxilo e hidroxilo, superóxido, singulete de oxígeno, y peroxinitrito (Nishikimi, 1975; Bodannes y Chan, 1979; Machlin y Bendich, 1987; Vatassery, 1996). Otra función importante de la vitamina C es la regeneración de vitamina E (Chan, 1993). Aunque las reacciones del ácido ascórbico ocurren en la fase acuosa, este puede evitar la oxidación de vitamina E (α-tocoferol), la cual detiene la peroxidación de las membranas (Seregi y cols., 1978; Niki, 1991). Se ha demostrado que la administración de ácido ascórbico disminuye la pérdida neuronal en jerbos con oclusión bilateral de las arterias carótidas (Stamford y cols., 1999) y el volumen del infarto en monos con oclusión transitoria de la arteria cerebral media (Henry y Chandy, 1998).

viii. Ácido lipoico (LA) y su derivado ácido dihidrolipoico (DHLA). Se trata de dos antioxidantes potentes que cruzan la barrera hematoencefálica (Packer y cols., 1997). Tienen la propiedad de atrapar especies reactivas, incluyendo anión superóxido, radical hidroxilo, singulete de oxígeno, óxido nítrico y peróxido de hidrógeno. Ambos, pero especialmente el DHLA, tienen actividad quelante de metales libres. El DHLA también recicla a otros antioxidantes (tales como vitamina C y vitamina E), incrementa los niveles intracelulares de glutation, y modula la actividad de factores de transcripción, especialmente la de NF-kB (Packer y cols., 1997; Packer, 1998). Se ha demostrado que el pretratamiento con 100 mg de DHLA o de LA reduce el volumen del infarto en ratones (Prehn y cols., 1992; Backhaus y cols., 1992) y en jerbos (Cao y Phillis., 1995) sometidos a isquemia cerebral global y/o focal.

Panigrahi y sus colaboradores, usando ratas pretratadas con ácido lipoico (25 mg/kg) y sometidas a oclusión bilateral de las carótidas comunes más hipotensión, encontraron reducción en la mortalidad de un 78 a un 26% a las 24 horas; este fármaco también evitó la pérdida de glutation en la corteza, estriado e hipocampo (Panigrahi y cols., 1996).

ix. Fenilbutilnitrona (PBN). Fármaco antioxidante sintético capaz de atrapar radicales libres de oxígeno y con base de carbono (Kotake, 1999). Se ha encontrado que la administración previa de PBN, aumenta la sobrevida (Carney y Floyd, 1991), disminuye el volumen del infarto después de la oclusión transitoria de la arteria cerebral media (MCA, por sus siglas en inglés) en ratas (Zhao y cols., 1994), y mejora la recuperación del estado energético cerebral (Folbergrova y cols., 1995). También reduce la necrosis neuronal en la neocorteza cuando se administra 30 min postisquemia, pero no cuando se administra antes o 6 horas después del evento isquémico (Pahlmark y Siesjo, 1996). También se ha observado que la PBN disminuye la disfunción mitocondrial cuando se administra 1 h después de la isquemia focal transitoria en ratas (Kuroda y cols., 1996). Schultz y colaboradores (1997) reportaron que la administración de PBN (25 mg/kg i.v.) 5 min antes y 30 min después de la oclusión de la MCA en ratas protege al endotelio vascular y, por tanto, aumenta la reperfusión postisquémica. No se tienen estudios sobre su toxicidad.

b. Fracaso de los antioxidantes en pacientes con EVC aguda

a.Tirilazad. Molécula derivada de los glucocorticoides sin actividad glucocorticoide o mineralocorticoide. Es un atrapador de radicales peroxilo e inhibe la lipoperoxidación dependiente de hierro (ec. 6 y 7) (Hall, 1995; Hall, 1997; Kavanagh y Kam, 2001). Se ha demostrado que tirilazad disminuye el volumen del infarto en ratas (Hall y Braughler, 1989; Xue y cols., 1992; Beck y Bielenberg, 1991; Park y Hall, 1994; Hall, 1995) y en gatos (Silvia y cols., 1987) sometidos a oclusión permanente o transitoria de la arteria cerebral media. Además, aumenta la sobrevida de jerbos sometidos a tres horas de oclusión unilateral de la arteria carótida (Hall y cols., 1988). Sin embargo, los ensayos clínicos de fase III no mostraron mejoría del estado funcional de pacientes con EVC aguda tratado con este fármaco (RANTTAS Investigators, 1996; Haley, 1998).

b. Ebselen. Es un antioxidante orgánico que contiene selenio. Se ha encontrado que su mecanismo de acción principal es mimetizar la acción de la peroxidasa del glutation, la cual elimina a las especies reactivas H_2O_2 y LOOH (Muller y cols., 1984; Maiorino y cols., 1988; Thomas y cols., 1990; Krinsky, 1992). También tiene efectos inhibitorios sobre las enzimas

ciclooxigenasa, lipooxigenasa y NADPH oxidasa (Schewe, 1995). Recientemente se ha reportado que el mecanismo de acción predominante podría ser vía el sistema tioredoxina más que el sistema del glutation (Zhao y cols., 2002). En el modelo de oclusión de la MCA, la administración 30 mg/kg ebselen reduce hasta un 53% el volumen del infarto (Dawson *y* cols., 1995) y la infusión intravenosa (1 mg/kg/h) reduce en un 28% el volumen del infarto (Imai y cols., 2003). Sin embargo, su efecto protector en pacientes fue poco claro al primer mes después del tratamiento, y no fue evidente en las evaluaciones neurológicas efectuadas al tercer mes; aunque un análisis posterior mostró cierta mejoría en un subgrupo de pacientes. Por ello, este fármaco continúa en estudio clínico (Saito y cols., 1998; Yamaguchi y cols., 1998; Gilgun-Sherki y cols., 2002).

c. Superóxido dismutasa (SOD). Esta enzima convierte el superóxido en peróxido de hidrógeno y representa la primera línea de defensa contra la toxicidad de las especies reactivas de oxígeno. Existen reportes de que la SOD atenúa el daño isquémico en jerbos con oclusión temporal de las arterias carótidas comunes (Tagaya y cols., 1992). Sin embargo, en un ensayo aleatorizado multicéntrico, la administración de superóxido dismutasa no mostró mejoría del resultado funcional en pacientes con daño cerebral severo (Young y cols., 1996).

d. Lubeluzol. Se ha informado que en el modelo de isquemia cerebral anterior más hipotensión, el tratamiento con lubeluzol, un modulador a la baja de la vía del ON, aumenta el número de neuronas viables (Haseldonckx *y* cols.*,* 1997). Sin embargo, en pacientes con EVC no mejoró el estado funcional ni la mortalidad a los tres meses del tratamiento (Diener, 1998; Grotta, 1997; Diener, 1999).

En resumen, ningún antioxidante, como cualquier otro neuroprotector potencial, ha probado ser eficaz en humanos con EVC aguda (Corbett y Nurse, 1998; de Keyser y cols., 1999; Gilgun-Sherki y cols., 2002; Green y cols., 2003).

2.4 Modelos tradicionales de isquemia cerebral

En vista de que la enfermedad cerebrovascular isquémica es un gran problema de salud pública, durante los últimos 35 años se han realizado numerosas investigaciones para conocer la fisiopatología del evento isquémico y para validar su posible tratamiento. El conocimiento actual sobre el daño neuronal durante y después de la isquemia cerebral está basado en modelos animales de isquemia cerebral (Juurlink y Sweeney, 1997; Dirnagl y cols., 1999; Lipton, 1999; Onténiente y cols., 2003). El conocimiento fisiopatológico abrió la posibilidad de la manipulación farmacológica útil para limitar el daño neuronal y reducir las deficiencias

funcionales.

Los modelos experimentales desarrollados, que habitualmente utilizan roedores (jerbos, ratas, ratones), se pueden agrupar en dos grandes categorías: modelos de isquemia focal y modelos de isquemia global. La isquemia focal resulta de la oclusión de una arteria específica, usualmente de la arteria cerebral media (MCA). La oclusión puede ser permanente o transitoria (remoción del bloqueo para permitir la reperfusión) (Hunter y cols., 1995; Green y cols., 2003). Diversos autores señalan que este modelo tiene mayor relevancia para la enfermedad cerebrovascular (Hunter y cols., 1995), lo que parece razonable, dado que en su mayoría los eventos cerebrovasculares son focales.

Los modelos de isquemia global, comúnmente del cerebro anterior, involucran frecuentemente un bloqueo transitorio (5-30 min) de las arterias carótidas comunes, lo que afecta áreas cerebrales muy extensas, especialmente las más vulnerables a los procesos isquémicos como el sector CA1 y CA3 del hipocampo, al caudoputamen y las capas 3, 5 y 6 de la corteza (Hossmann, 1993). Es interesante señalar que existe una vulnerabilidad temporal y espacial dentro de la región CA1. Las neuronas piramidales mueren en una forma ordenada, de medial a lateral y de septal a temporal (Pulsinelli y cols., 1982). Los mecanismos subyacentes de esta vulnerabilidad son desconocidos, pero no parecen correlacionar con la vasculatura del hipocampo (Marinkovic y cols., 1992). Los modelos de isquemia global tienen la característica de mantener el flujo sanguíneo del tallo cerebral, permitiendo a los animales la habilidad de ventilar espontáneamente. Aunque existen muchas maneras de inducir interrupción del flujo sanguíneo al cerebro anterior, en la mayoría de los modelos incluye oclusión de las arterias carótidas comunes.

El modelo global más simple, y por lo tanto el más popular, es el modelo de oclusión bilateral de las arterias carótidas comunes en jerbos. Esta oclusión es suficiente para producir isquemia severa del cerebro anterior, debido a que los jerbos carecen de arterias comunicantes posteriores, necesarias para completar el círculo de Willis el cual, en humanos y en ratas, permite el flujo sanguíneo colateral (Levine y Sohn, 1969).

Los modelos de isquemia global en ratas incluye la oclusión de dos (carótidas) o cuatro (carótidas más vertebrales) vasos. El primero comprende la oclusión simultánea de ambas carótidas y requiere de hipotensión para producir isquemia. El segundo procedimiento se lleva a cabo en dos fases, en una primera fase se ocluyen las arterias vertebrales y, en la segunda, se ocluyen simultáneamente las carótidas. Tienen la ventaja de que en la segunda fase el evento isquémico se realiza en un animal conciente y en movimiento (Hunter y cols., 1995).

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En los estudios más recientes se ha dado preferencia al uso de ratones. En los ratones se observan diferencias claras en el sistema de irrigación, como presencia o no de la arteria comunicante posterior. Cuando esta arteria está ausente permite que la oclusión de las arterias carótidas comunes produzca menos del 23% de microperfusión cortical basal (Small y Buchan, 2000).

Aún cuando la oclusión arterial sea permanente, existe alguna recuperación del flujo a través de arterias colaterales, produciendo un territorio isquémico transitorio (zona de penumbra isquémica) (Small y Buchan, 2000). Los modelos de oclusión transitoria tienen todos los rasgos de la oclusión permanente, además de la complicación adicional del daño por reperfusión. El tejido isquémico reperfundido es tejido en riesgo, y la mejor representación del evento cerebrovascular en humanos, particularmente después de la trombolisis espontánea o terapéutica.

Algunos autores cuestionan el uso de modelos de isquemia global, el cual emula más a un paro cardiaco que a un evento vascular focal, pero la precisión con que el daño global isquémico induce daño en las neuronas da una modalidad precisa y cuantificable, que puede ser útil para valorar la eficacia de un agente neuroprotector. Además, la característica de mantener el flujo sanguíneo del tallo cerebral, lo hace diferente del paro cardíaco. Por otro lado, se tiene un gran número de fármacos capaces de reducir el daño producido por la isquemia focal, pero una revisión detallada de los últimos 10 años revela que pocos, si es que a alguno, de estos compuestos tienen efecto protector en modelos de isquemia cerebral global (Small y Buchan, 2000).

2. 4.1 Indicadores de daño isquémico y de eficacia farmacológica en los modelos tradicionales

En general, la determinación del daño se basa en parámetros morfológicos; esto es, conteo neuronal o determinación del volumen del infarto dentro de los primeros 7 días después del episodio isquémico (Corbett y Nurse, 1998). Se estima que la evaluación histológica es, en principio, incompleta porque: (a) centra la atención en el conteo de neuronas necróticas más que en células normales y, (b) existen áreas rara vez valoradas por su dificultad inherente de cuantificación (Corbett y Nurse, 1998). Además, no hay correlación entre la capacidad de un fármaco para reducir el daño morfológico (volumen del infarto) en modelos tradicionales de isquemia cerebral y los indicadores de eficacia en pacientes con EVC (de Keyser, 1999; Green y cols., 2003; Cheng y cols., 2004)). Por lo anterior, se ha cuestionado el valor del volumen del infarto como blanco relevante en los experimentos

animales para el desarrollo de la terapéutica del EVC (Corbett y Nurse, 1998).

2.4.2 Cuestionamientos metodológicos a los modelos tradicionales

Existen numerosos cuestionamientos que parecen explicar la falta de correlación entre los resultados de los estudios preclínicos y los ensayos clínicos:

i. Animales jóvenes. En los modelos tradicionales de isquemia cerebral, los investigadores usualmente eligen animales jóvenes y sanos, bajo condiciones de laboratorio rigurosamente controladas. Sin embargo, los pacientes con enfermedad cerebrovascular seleccionados para ensayo clínico, usualmente, son viejos (Wardlaw y cols., 1996) y, generalmente, presentan otras enfermedades crónicas, tales como ateroesclerosis, hipertensión, diabetes, hiperlipidemia (Cheng y cols., 2004; Onténiente y cols., 2003); las cuales pueden afectar el resultado funcional y alterar los indicadores de eficacia farmacológica (Demchuk y Buchan, 2001).

ii. Interrupción abrupta del flujo sanguíneo en cerebros previamente sanos. La interrupción del flujo sanguíneo suele ser transitorio, donde el inicio de la isquemia y su reversión son abruptas (Ginsberg y Busto, 1989). Por el contrario, en humanos el evento isquémico agudo sucede habitualmente en un cerebro con arterias dañadas por procesos de ateroesclerosis o arteriosclerosis; por lo tanto, crónicamente isquémico (Rodriguez y cols., 2000a).

iii. Administración del potencial neuroprotector antes del evento isquémico. En la mayoría de los estudios, aun cuando esto es cada vez menos frecuente, la administración del potencial neuroprotector es previa al evento isquémico (Grotta, 1995); mientras que en los ensayos clínicos la administración del fármaco se lleva a cabo después de iniciado el evento isquémico.

iv. Volumen del infarto como principal indicador de daño y neuroprotección. En la mayoría de los estudios preclínicos, el efecto neuroprotector se mide en términos de la capacidad para reducir el volumen del infarto y/o el grado de pérdida neuronal (Corbett y Nurse, 1998), y la mortalidad es ignorada, o al menos no reportada (Grotta, 1995). En contraste, en los ensayos clínicos, la eficacia neuroprotectora se mide con escalas de la función neurológica tales como la de los Institutos Nacionales de Salud (NIH Stroke Scale) y la de Rankin Modificada (Cheng y cols., 2004), que típicamente usan la sobrevida y parámetros funcionales, principalmente de la función motora. Además, se ha demostrado que el volumen del infarto correlaciona pobremente con el resultado funcional (Rogers y cols., 1992; Hunter y cols., 1995), ya que lesiones pequeñas en localizaciones críticas pueden producir gran déficit funcional y, al contrario, lesiones extensas en áreas relativamente silentes producen poca pérdida detectable

de la función (de Keyser, 1999; Green y cols., 2003; Cheng y cols., 2004).

v. Evaluación funcional incompleta. Algunos estudios recientes sobre neuroprotección en los modelos animales de isquemia cerebral han puesto más interés en la evaluación funcional. Se han desarrollado varias mediciones funcionales para estudio de los animales isquémicos (Hunter y cols., 1998). Sin embargo, no se ha documentado la validez de estas mediciones para detectar neuroprotección y algunos autores señalan que no correlacionan con las usadas en los ensayos clínicos (Cheng y cols., 2004). En la mayoría de los casos los investigadores eligen la prueba de acuerdo a su expertis, y no necesariamente porque la prueba represente aspectos importantes del resultado funcional en animales. En los ensayos clínicos, se usan escalas como indicadores para determinar el grado de daño (NIH Stroke Scale) y de discapacidad física o mental (Rankin Scale, Barthel Index) del paciente. Ninguna escala clínica se ha desarrollado para correlacionar con el tamaño del infarto o cualquier batería preclínica de pruebas (Cheng y cols., 2004).

vi. Descuido en el daño y la protección de la sustancia blanca. La mayor parte de los neuroprotectores descritos son capaces de reducir el daño tisular en la sustancia gris (cuerpos neuronales). Sin embargo, la isquemia y la reperfusión también producen daño axonal, y se ha considerado que una de las causas del fracaso de estos fármacos en la clínica es que no protegen del daño a la sustancia blanca (Dewar y cols., 1999), que en los humanos comprende un 50% del volumen cerebral. Al respecto, se ha señalado que los antioxidantes protegen del daño tanto al cuerpo neuronal como a la sustancia blanca (McCulloch y Dewar, 2001; Imai y cols., 2001).

Por otro lado, también se ha reportado que la falta de correlación entre los estudios de farmacología básica y clínica se pueden atribuir en parte a ensayos clínicos poco controlados, destacan los siguientes cuestionamientos: diseño inapropiado del ensayo clínico, incluyendo tamaño de la muestra, inclusión de pacientes fuera de la ventana terapéutica (más de 3-6 horas), administración de dosis menores a las efectivas en los modelos animales, y algunos ensayos se han terminado prematuramente por problemas de seguridad los cuales no se detectaron en los modelos preclínicos. Algunos otros problemas metodológicos como un desbalance en la distribución de variables clínicamente importantes, tales como severidad del evento vascular, edad del paciente y presencia de enfermedades coexistentes (Dirnagl y cols., 1999; de Keyser y cols., 1999; Onténiente y cols., 2003; Cheng y cols., 2004).

Como se ha mencionado previamente, los pacientes con enfermedad cerebrovascular

aguda presentan déficit marcado en las funciones cognitivas, sensoriales y motoras, además de un alto índice de mortalidad; en general existe consenso en usar la combinación de mortalidad e indicadores funcionales para determinar la eficacia de agentes neuroprotectores potencialmente útiles en la clínica (Hunter y cols., 1998; de Keyser y cols., 1999). Por esta razón, es deseable continuar en el desarrollo de modelos que se acerquen más a la enfermedad isquémica en humanos y considerar, primero, la obtención de una evaluación detallada de las deficiencias funcionales y, segundo, determinar si éstas son medidas sensibles al tratamiento farmacológico. El modelo experimental que parece guardar mayor relación con la EVC de humanos es el de la oclusión secuencial de las arterias carótidas comunes, desarrollado por Rodriguez y colaboradores (2000a).

2.5 Modelo de sección secuencial de las arterias carótidas comunes (SSACC).

La SSACC es un modelo de isquemia global incompleta. La isquemia se produce porque se interrumpe de manera secuencial la circulación sanguínea del sistema carotídeo, dejando intacta la circulación del sistema basilar encargada de irrigar a los centros reguladores de funciones vitales como la respiratoria y la cardiovascular. El procedimiento contempla dos etapas; en la primera, se liga y secciona la carótida común izquierda; treinta y dos días después (segunda fase) se secciona la carótida contralateral. Este modelo tiene diferencias básicas con los modelos tradicionales. En principio, utiliza animales viejos, que han mostrado ser más susceptibles al daño isquémico (Yager y cols., 1996; Fuentes-Vargas y cols., 2002). El intervalo que existe (32 días) entre la oclusión de una y la otra carótida hace posible que el fenómeno isquémico agudo se desarrolle en un cerebro con perfusión crónicamente reducida, en analogía a pacientes con EVC que, previo a un evento isquémico mayor, han sufrido ataques isquémicos transitorios. Para determinar el grado de daño neurológico, este modelo utiliza la mortalidad y, a través de un examen clínico minucioso, cuantifica las alteraciones neurológicas producto de la interrupción del flujo sanguíneo. La mortalidad y la severidad del déficit neurológico son los indicadores utilizados para determinar la eficacia del tratamiento farmacológico. Cabe agregar que el daño tisular concuerda con lo reportado para otros modelos de isquemia del cerebro anterior; bilateralmente afecta grandes áreas, incluyendo las áreas más vulnerables como hipocampo, caudoputamen y corteza (Rodriguez y cols, 2000a). Con el empleo de este modelo se demostró que el dexrazoxano, un agente quelante, tiene efectos neuroprotectores excepcionales (Rodriguez y cols., 2000b; Rodriguez y cols., 2003b).

Cabe agregar que la sección de la primera carótida da lugar a un aumento progresivo del diámetro de las arterias que conforman el círculo de Willis, indicando que la disminución del

flujo cerebral se compensa por el aumento en el diámetro de las arterias (Rodriguez y cols., 2000a). En otros modelos, se ha reportado que episodios breves de isquemia cerebral confieren resistencia a un evento subsecuente de isquemia más prolongada que, de otra manera, produciría mayor daño. Este fenómeno es conocido como precondicionamiento o tolerancia isquémica (Dirnagl y cols., 2003), y es una reacción aguda y/o crónica a estímulos nocivos como la isquemia cerebral global (Kitagawa y cols., 1990) o focal (Stagliano y cols., 1999), y a radicales libres de oxígeno (Wiegand y cols., 1999). Ante estos estímulos, el cerebro responde con inducción de mecanismos protectores, principalmente en la zona de penumbra (Dirnagl y cols., 2003). En minutos, hay activación de mecanismos antiexcitotóxicos que incluyen la liberación de GABA y adenosina, activación de canales de K^+ dependientes de ATP; en horas, activación de mecanismos antiapoptóticos y antiinflamatorios como IL-10, proteínas Bcl, eritropoyetina, factores de transcripción a través del factor inducible por hipoxia (HIF-1); en días y semanas, activación de mecanismos de regeneración y reparación como vasculogénesis y neurogénesis. Basados en este conocimiento, se puede inferir que la isquemia cerebral aguda (corte de la carótida contralateral) se da en un cerebro que expresa tolerancia isquémica. La sección secuencial de las arterias carótidas comunes con un intervalo de 2, 4, y 16 días muestra una mortalidad del 100% en algunas horas. Sin embargo, la oclusión con un lapso de 32 días posiblemente desarrolla un suministro colateral máximo y generación de nuevos vasos (Rodriguez y cols., 2000a). Este fenómeno quizá se explica, en parte, por la resistencia que da un evento isquémico leve (sección de la primera carótida). El mecanismo molecular del precondicionamiento isquémico da claves de mecanismos protectores endógenos, tales como la nueva expresión de genes de expresión temprana (c-fos, c-jun, jun-B, jun-D), proteínas de choque térmico (hsp-70), enzimas antioxidantes como superóxido dismutasa de manganeso (Bordet y cols., 2000), catalasa, glutation peroxidasa; inhibidores de la metaloproteínasas (Bcl-2 y Bcl-X$_L$) (Neumar, 2000).

Basados en estos hallazgos, el intervalo de 32 días entre la oclusión de las carótidas hace posible una condición basal compleja y da un margen conveniente de sobrevida para estudiar fármacos que modifiquen tanto el patrón de sobrevida como el déficit neurológico.

III. RAZONAMIENTO CIENTÍFICO

3.1   Planteamiento del problema

La información disponible señala que la isquemia/reperfusión cerebral da lugar a la formación excesiva de especies reactivas, las cuales presumiblemente juegan un papel central en el daño neuronal, al incrementar la lipoperoxidación en las membranas celulares y oxidar otras biomoléculas esenciales como ADN y proteínas. Asimismo, que los antioxidantes tienen un efecto neuroprotector considerable, ya que disminuyen el volumen del infarto en los modelos experimentales tradicionales de isquemia cerebral. Sin embargo, no se tiene información sobre su capacidad de reducir la mortalidad y las alteraciones neurológicas derivadas de la isquemia cerebral.

3.2   Pregunta científica

¿Pueden los antioxidantes disminuir la mortalidad y las alteraciones neuroconductuales en el modelo de SSACC?

3.3   Hipótesis

La administración de ácido ascórbico, ácido dihidrolipoico, t-butilhidroquinona, o fenilbutilnitrona disminuye la mortalidad y las alteraciones neuroconductuales producidas por la SSACC.

3.4   Estrategia experimental

Desarrollar un sistema de evaluación y cuantificación como indicadores tanto del daño isquémico, así como de neuroprotección en el modelo de SSACC.

3.5   Objetivo general

El objetivo primario de este trabajo fue determinar si la administración de ácido ascórbico, ácido dihidrolipoico, t-butilhidroquinona o fenilbutilnitrona disminuye la mortalidad y la discapacidad neurológica producidos por la sección secuencial de las arterias carótidas comunes (SSACC).

3.6   Objetivos específicos

1. Determinar el patrón de mortalidad de los animales sometidos a la SSACC.
2. Caracterizar el déficit neurológico de los animales sometidos a la SSACC.
3. Determinar el efecto del ácido ascórbico, ácido dihidrolipoico, t-butilhidroquinona, y fenilbutilnitrona sobre el patrón la sobrevida y el déficit neurológico producida por la SSACC.

IV. MATERIAL Y MÉTODOS

4.1 Animales

En todos los experimentos se utilizaron ratones machos envejecidos (40 a 60 semanas de edad), de la cepa CFW (obtenidos inicialmente de la casa Taconic Farms, Germantown, NY), de 38 a 55 g de peso corporal, obtenidos del Bioterio de la Facultad de Medicina de la UNAM. Se colocaron de 3 a 5 animales (de la misma camada) por jaula en un cuarto de temperatura controlada (22 ± 2 °C, humedad relativa 55 ± 3%), con un ciclo de luz-oscuridad normal (luz de 8 a.m. a 8 p.m.), con libre acceso al agua y al alimento (Purina Chow, St. Louis, MO, USA). Se permitió que los ratones se aclimataran a las condiciones ambientales por al menos una semana previa a los experimentos. Doce horas antes del procedimiento quirúrgico, se les retiró el alimento y se mantuvo libre el acceso al agua. La cirugía y las evaluaciones conductuales se realizaron entre las 09:00-14:00 horas. Todos los experimentos realizados se adhirieron a la ética experimental de la Declaración de Helsinki y a lo establecido en el Reglamento de la Ley General en Materia de Investigación para la Salud de México (Secretaria de Salud, 1987).

4.2 Experimentos preliminares

En un grupo de experimentos preliminares estudiamos la mortalidad y las alteraciones conductuales producidas por la sección secuencial de las arterias carótidas comunes (SSACC), procedimiento que se describe en detalle en la sección de experimentos finales.

a. Mortalidad. Después de la segunda cirugía, se registró continuamente el número de muertes durante las primeras 6 horas y, después, cada 6 horas durante las primeras 24 horas y cada 12 horas hasta el fin del experimento (72 h). Estos datos se utilizaron para calcular las curvas de sobrevida.

No se observaron muertes en los animales control (sin anestesia ni cirugía) y, excepto en un caso (1/25), no se observaron muertes en los ratones sham. Por lo tanto, los animales sham mostraron 96% de sobrevida. Después de la segunda cirugía, 10% (5/50) de los animales murieron dentro de los primeros 15 min, y el número de muertes aumentó progresivamente, de 18% (9/50) a los 60 minutos a 52% (26/50) a las 24 horas, 62% (31/50) a las 48 horas, 64% (32/50) a las 72 horas. La figura 3 muestra las curvas de sobrevida de Kaplan-Meier para los animales sham y los ratones experimentales sometidos a la SSACC. Las proporciones de sobrevida a las 24, 48 y 72 horas para los ratones con SSACC fueron de 48, 38, y 36%, respectivamente. Los resultados de la prueba de rangos

logarítmicos indicaron diferencias estadísticas altamente significativas entre las dos curvas (p<0.0001).
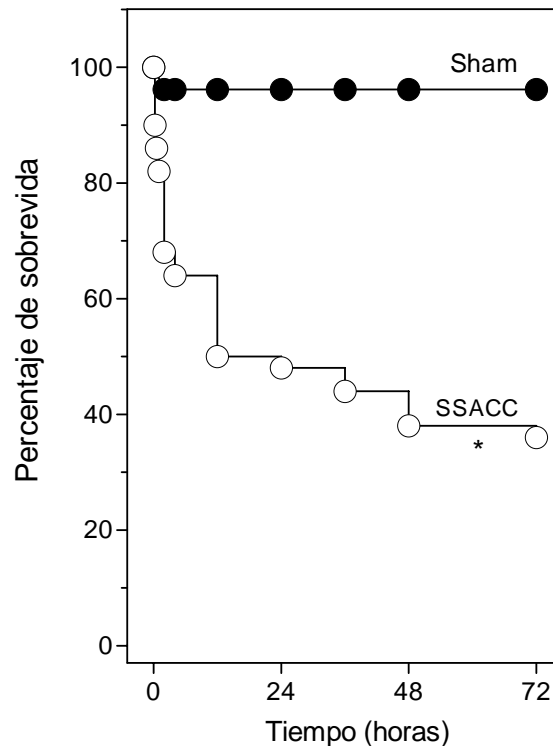
Figura 3. Curvas de sobrevida en ratones del grupo control (sham, ●) y en ratones sometidos a la SSACC (○). El registro de las muertes fue continuo en las primeras 6 horas después de la segunda cirugía, cada 6 horas durante las primeras 24 h, y después, cada 12 h hasta el fin del experimento (72 h). Las abscisas representan el tiempo después de la segunda cirugía, la ordenadas el porcentaje de sobrevida. n = 25 en el grupo sham y 50 en el grupo con SSACC al inicio de los experimentos. Las curvas fueron calculadas por el método de Kaplan-Meier y comparadas usando la prueba de rangos logarítmicos (*p<0.0001).

b. Alteraciones neuroconductuales. El procedimiento utilizado para la evaluación neurológica es una adaptación del validado por Irwin (1968), el cual se describe con detalle en la sección de experimentos finales. Estas evaluaciones se realizaron inmediatamente antes (basal) y 24, 48 y 72 h después de la segunda cirugía. En esta fase, para cada animal, se realizó un examen neurológico minucioso con el objetivo de identificar todas las alteraciones inducidas por la SSACC. Se tomó como presente a la manifestación de determinada alteración en algún momento del periodo de observación (24, 48 o 72 h después de la segunda cirugía).

Excepto por la presencia de ptosis (92%), ninguno de los animales control (sham) mostró anormalidades neurológicas, mientras que en los ratones con SSACC se detectó la presencia de un gran número de alteraciones (Cuadro 1). Con estos datos se determinó la frecuencia de las alteraciones neuroconductuales producidas por la SSACC.

Cuadro 1. Etapas del examen neurológico

Fase de observación*	Fase de manipulación*
Hipomotilidad	Pasividad
Posición corporal aplanada	Hiperreactividad
Posición corporal lateralizada	Irritabilidad
Encorvamiento	Ptosis
Piloerección	Incontinencia urinaria
Marcha anormal	Disminución del tono corporal
Desplazamiento en círculo	Flexión de extremidad anterior
Temblor	Disminución de la fuerza muscular
Sacudidas	Rotación corporal
Convulsiones	Incoordinación motora
Dificultad respiratoria	- plano inclinado
	- cuerda tirante
	Hipoalgesia
	Hiperalgesia

* Listados en el orden que fueron evaluados

La figura 4 muestra la frecuencia de las alteraciones neuroconductuales producidas por la SSACC. Las alteraciones más consistentes producidas por la SSACC fueron, en el orden que se indica: incoordinación motora, postura lateralizada, hipomotilidad, disminución del tono corporal, temblor, encorvamiento, pasividad, disminución de la fuerza muscular, flexión de la extremidad anterior, postura aplanada, marcha anormal, desplazamiento en círculo, piloerección, dificultad respiratoria. Además, en algunos animales ($<5\%$), se notó hipermotilidad, estereotipia, aumento del tono muscular, catatonia, diarrea. Con base en estas observaciones se llevaron a cabo los experimentos finales.

FRECUENCIA (%). 24-48-72 h

ALTERACIONES NEUROLÓGICAS

1. PTOSIS
2. INCOORDINACIÓN MOTORA
3. POSTURA LATERALIZADA
4. HIPOMOTILIDAD
5. DISMINUCIÓN DEL TONO CORPORAL
6. TEMBLOR
7. ENCORVAMIENTO
8. PASIVIDAD
9. DEBILIDAD MUSCULAR
10. FLEXIÓN DE LA EXTREMIDAD ANTERIOR
11. POSTURA CORPORAL APLANADA
12. MARCHA ANORMAL
13. MUERTE
15. DESPLAZAMIENTO EN CÍRCULO
14. INCONTINENCIA URINARIA
16. PILOERECCIÓN
17. DIFICULTAD RESPIRATORIA
18. HIPOALGESIA
19. IRRITABILIDAD
20. ROTACIÓN CORPORAL
21. CONVULSIONES
22. SACUDIDAS
23. HIPERALGESIA
24. HIPERREACTIVIDAD

Figura 4. Frecuencia acumulada de las alteraciones neuroconductuales detectadas a lo largo de todo el periodo de observación en ratones sometidos a la SSACC. La evaluación neurológica involucró una fase inicial de observación seguida de una fase de manipulación durante la cual cada animal fue sometido a una secuencia de manipulaciones, iniciando con el estímulo menos perturbador. Los animales fueron examinados antes (basal) y 24, 48 y 72 horas después de la segunda cirugía. *n* = 50 al inicio de los experimentos.

4.3   Experimentos finales

a. Procedimiento para producir isquemia cerebral aguda. Como ya se mencionó, la isquemia cerebral fue producida por el procedimiento de sección secuencial de las arterias carótidas comunes (SSACC) (Rodriguez y cols., 2000a). Brevemente, el procedimiento consistió en realizar bajo anestesia leve con éter, una incisión en la cara anterior del cuello, separar cuidadosamente la arteria carótida común (ACC) izquierda del nervio vago y, excepto el grupo sham, seccionar entre dos ligaduras, y cerrar la incisión con hilo quirúrgico. Después de la cirugía, los animales fueron colocados en un área de recuperación, manteniendo su temperatura corporal con lámparas. Una vez que los animales se recuperaron de la anestesia se regresaron a sus jaulas originales. Treinta y dos días después, se realizó una evaluación clínica usando el procedimiento descrito más abajo. Los ratones que mostraron algún déficit neuroconductual fueron excluidos de los experimentos posteriores. La muestra de animales se asignó de manera aleatoria a diferentes grupos experimentales antes de la anestesia y sección de la carótida común derecha. Como grupos control se utilizaron ratones sin anestesia ni cirugía, y ratones con anestesia y procedimiento quirúrgico, excepto ligadura y sección de la carótida derecha (sham).

b. Registro de la mortalidad producida por la SSACC. Después de la segunda cirugía, se registró continuamente el número de muertes durante las primeras 6 horas y, después, cada 6 horas durante las primeras 24 horas; y cada 12 horas hasta el fin del experimento (72 h). Estos datos se utilizaron para calcular las curvas de sobrevida.

c. Evaluación neurológica. Como ya se mencionó, el examen neurológico de los ratones se llevó a cabo utilizando una modificación del procedimiento validado por Irwin (1968). Este examen involucra una fase inicial de observación seguida por una fase de manipulación (Cuadro 2). Durante esta última, cada animal fue sometido a una serie de manipulaciones, iniciando con las de estímulos menos perturbadores. Los ratones se evaluaron individualmente. El día del experimento cada animal se colocó en el centro de un área con 3 paredes (madera, 60X60X20 cm) y el examen neurológico se realizó antes de administrar la anestesia para la segunda cirugía, y 24, 48 y 72 horas posteriores a la misma. El mismo observador, sin conocimiento previo de los tratamientos respectivos, condujo todas las evaluaciones neurológicas. El Cuadro 2a y 2b describe los criterios utilizados para determinar la presencia de alteraciones neuroconductuales.

Cuadro 2a. Criterios utilizados en la fase de observación

Parámetro	*Descripción*

Hipomotilidad. Disminución de la motilidad espontánea del ratón experimental comparado con el control externo cuando se colocó sobre la mesa de exploración. Calificado como 1, 2 o 3 en términos de su locomoción, de la velocidad y vigor de sus movimientos.

Postura aplanada. Posición corporal anormal caracterizada por movimientos lentos y arrastrar el cuerpo al caminar sobre la mesa de exploración.

Postura lateralizada. Posición corporal anormal caracterizada por la tendencia persistente a reclinarse sobre un costado.

Encorvamiento. Presencia persistente de una postura encorvada.

Piloerección. Elevación persistente del pelo del dorso.

Marcha anormal. Tendencia a caminar en zig-zag, con balanceo, o sacudidas cuando el animal avanza.

Deplazamiento en círculo. Marcha persistente hacia un lado, espontánea o forzada con un empujón suave con un dedo del observador.

Temblor. Presencia de movimientos oscilantes, finos y repetitivos.

Sacudidas. Movimientos corporales abruptos.

Convulsiones. Sacudidas repetitivas seguidas por extensión de las extremidades.

Dificultad respiratoria. Presencia de movimientos respiratorios exagerados e irregulares acompañados por sonidos respiratorios.

Cuadro 2b. Criterios utilizados en la fase de manipulación

Pasividad. Disminución de la respuesta conductual cuando los animales son cubiertos con la mano para restringir sus movimientos.

Disminución del tono corporal. Disminución de la resistencia a la compresión o flacidez de los músculos abdominales, determinado por una compresión suave de las paredes abdominales usando los dedos pulgar e índice.

Flexión de la extremidad anterior. Incapacidad para extender una de las extremidades anteriores cuando el animal es suspendido a 10 cm y lentamente bajado para observar la simetría en las extremidades mientras el ratón intenta alcanzar una rejilla de alambre.

Debilidad muscular. Disminución de la resistencia cuando el animal es colocado sobre una rejilla de alambre y jalado suavemente por la cola.

Incoordinación motora. Disminución en la capacidad para moverse y permanecer por lo menos 10 seg sobre un plano inclinado (45°) y/o para agarrarse a la cuerda tirante (30 cm arriba de la plataforma) con sus extremidades y cola, y para permanecer por al menos 10 seg. Calificados como 1, 2 o 3 en términos de persistencia, vigor y coordinación de sus movimientos.

Excepto en dos parámetros, la ausencia/presencia de cualquiera de estas alteraciones fue calificada con 0 o 1 (0 = no presente, 1 presente). La motilidad espontánea y la coordinación motora fue calificada en términos de su nivel de afectación sobre la escala 0-3 (0 = normal, 1

= disminución leve, 2 = disminución marcada, 3 = total incapacidad para moverse. Usando esta estrategia, el examen neurológico de cada animal se realizó en un lapso de 3 o 4 min.

d. Escala de discapacidad neurológica (EDN). De las 24 alteraciones detectadas en los experimentos preliminares, se seleccionaron sólo 16 por ser las más consistentes y características. Con estos datos se diseñó una escala para determinar el grado o nivel de discapacidad funcional (Cuadro 3). Los detalles sobre la escala usada para determinar el grado de discapacidad funcional después de la isquemia cerebral ya la hemos reportado [Rodriguez y cols., 2003a; Rodriguez y cols., 2005). Brevemente, la EDN comprende 10 pasos progresivos, inicia en 0 (normal) y se extiende hasta 10 (muerte debida a SSACC). En esta escala el mayor puntaje indica disfunción neurológica más severa. Consecuentemente, los seis grados principales indican: cero (normal) significa sin disfunción neurológica, y 2 disminución leve en la motilidad y la presencia de pasividad. El nivel 4 representa disfunción neurológica moderada e incluye hallazgos tales como postura aplanada y/o lateralizada, encorvamiento, marcha atáxica, temblor, disminución del tono, debilidad muscular, incoordinación motora leve. El nivel 6 representa hipomotilidad moderada, desplazamiento en círculo, sacudidas, convulsiones, flexión de extremidad anterior, incoordinación moderada. El 8 indica incapacidad total para moverse, incoordinación severa, dificultad respiratoria. El nivel 10 corresponde a muerte debida a SSACC. En los casos cuando no se alcanzó un nivel preciso se tomó el número más cercano (1, 5, 7, 9).

Cuadro 3. Escala de discapacidad neurológica (EDN).

0 = Normal	6 = Hipomotilidad 2
2 = Hipomotilidad 1 Pasividad	Desplazamiento en circulo Sacudidas/convulsiones Flexión de extremidad anterior Incoordinación motora 2
4 = Postura corporal aplanada Postura lateralizada Encorvamiento Marcha anormal Piloerección Temblor Tono corporal disminuido Debilidad muscular Incoordinación motora 1	8 = Hipomotilidad 3 Incoordinación motora 3 Dificultad respiratoria 10 = Muerte

4.4 Estudio del efecto de los antioxidantes seleccionados sobre la mortalidad y déficit neurológico producido por la SSACC

a. Procedimiento metodológico. Para determinar el efecto de los antioxidantes seleccionados, se utilizó un lote grande de animales con sección previa de la carótida común izquierda y se distribuyó al azar en varios grupos experimentales. Cinco grupos (n = 18-22) fueron sometidos a anestesia y sección de la arteria carótida común derecha. Un grupo control (sham, n = 14) fue sometido a anestesia y procedimiento quirúrgico completo, excepto ligadura y sección de la arteria, y otro grupo de ratones control-control (n = 10) que no fueron sometidos ni a la anestesia ni a la cirugía.

Quince min después de la segunda sección arterial, se administró por vía i.p. ácido ascórbico (500 mg/kg), ácido dihidrolipoico (100 mg/kg), t-butilhidroquinona (t-BHQ; 100 mg/kg) o fenilbutilnitrona (PBN; 100 mg/kg). Los grupos control recibieron solución salina. Las muertes fueron registradas a los tiempos indicados y los sobrevivientes se evaluaron como se describió antes. Las dosis usadas de los antioxidantes son las reportadas como eficaces para limitar el volumen del infarto en otros modelos de isquemia cerebral (Murphy y cols., 1991; Prehn y cols., 1992; Stamford y cols., 1999; Yang y cols., 2000).

Usamos dos procedimientos para cuantificar el efecto de los antioxidantes sobre las alteraciones neuroconductuales producidas por SSACC: evaluación neurológica y motilidad espontánea. El día de la prueba, la secuencia fue: motilidad espontánea y evaluación neurológica. Todos los experimentos fueron realizados en una habitación semi-oscura y silenciosa. En cada ensayo, los animales fueron evaluados en orden aleatorio. El mismo observador, sin conocimiento previo de los tratamientos respectivos, condujo todas las evaluaciones neurológicas.

i. Evaluación neurológica. Las observaciones y manipulaciones estuvieron dirigidas a identificar la presencia o ausencia de los elementos más consistentes y característicos (Cuadro 2) del síndrome isquémico inducido por SSACC: hipomotilidad, pasividad, postura aplanada, postura lateralizada, encorvamiento, marcha anormal, piloerección, temblor/sacudidas/convulsiones, dificultad respiratoria, desplazamiento en círculos, disminución del tono corporal, debilidad muscular, parálisis de la extremidad anterior, e incoordinación motora (descritos previamente).

ii. Motilidad espontánea. La motilidad espontánea se midió con un equipo de actividad locomotora (Columbus instruments, Columbus OH) y sólo se registraron los movimientos de

la actividad horizontal (desplazamiento) de los animales. La caja de actividad locomotora (43.2 x 44.4 x 20 cm, con paredes y techo de acrílico transparente) está equipada con sensores de luz infraroja sobre cada eje (espaciados, 2.65 cm; diámetro del rayo, 0.32 cm). Los contadores externos registran todas las interrupciones de los haces de luz de cualquiera de los sensores. Los ratones se probaron individualmente en un cuarto semi-oscuro y silencioso. Los ratones se tomaron de sus jaulas y se colocaron en el centro del campo y se conectó inmediatamente el contador mecánico. La prueba se realizó inmediatamente antes (basal) y a las 24, 48 y 72 horas después de la oclusión de la carótida derecha. Las cuentas de la motilidad son el número de interrupciones en una sesión de 1 min.

iii. Peso corporal. Los animales se pesaron inmediatamente antes (basal) y 24, 48 y 72 horas después de la segunda cirugía.

4.5   Fármacos antioxidantes

Todos los compuestos fueron obtenidos de Sigma, Saint Louis MO, USA. Los fármacos fueron preparados inmediatamente antes de su empleo y disueltos en solución salina al 0.9%. La administración de los fármacos fue en un volumen de 0.1 ml/10 g de peso.

4.6   Análisis estadístico

Las curvas de sobrevida fueron calculadas con el método de Kaplan-Meier y comparadas usando la prueba de rangos logarítmicos (2 colas). Los puntajes de discapacidad neurológica para los diferentes tratamientos y a los diferentes tiempos (24, 48, y 72 horas) fueron comparados contra su respectivo grupo control con solución salina usando la prueba de Kruskal-Wallis (análisis de varianza no paramétrico) seguido por la prueba de comparaciones múltiples de Dunn. Los datos de la motilidad y del peso corporal se analizaron con ANOVA, seguida por la prueba de Dunnett para comparar los grupos que recibieron tratamiento antioxidante con el de solución salina. En todos los casos, un valor de probabilidad de 0.05 indicó significancia estadística. Los análisis fueron realizados usando el software GraphPad Prism versión 3.02 para Windows (GraphPad Software, San Diego, CA).

V. RESULTADOS

5.1   Mortalidad

En este grupo de experimentos no se observaron muertes en los animales control (sin anestesia ni cirugía) y sólo se observó una en los ratones del grupo sham (1/14). Por lo tanto los animales sham mostraron 93% de sobrevida a las 72 h (Fig. 5).

Después de la segunda cirugía, 5% de los animales murieron dentro de los primeros 15 min, y el número de muertes aumentó progresivamente, de 10% a los 60 min a 43% a las 24 horas, 67% a las 48 horas, 67% a las 72 horas. La figura 5 compara las curvas de sobrevida de Kaplan-Meier para los animales sham y los ratones experimentales sometidos a SSACC. Los índices de sobrevida a las 24, 48 y 72 h para los ratones con SSACC fueron de 57%, 33%, y 33%, respectivamente. Los resultados de la prueba de rangos logarítmicos indican diferencias estadísticas altamente significantes entre las dos curvas (p<0.001).
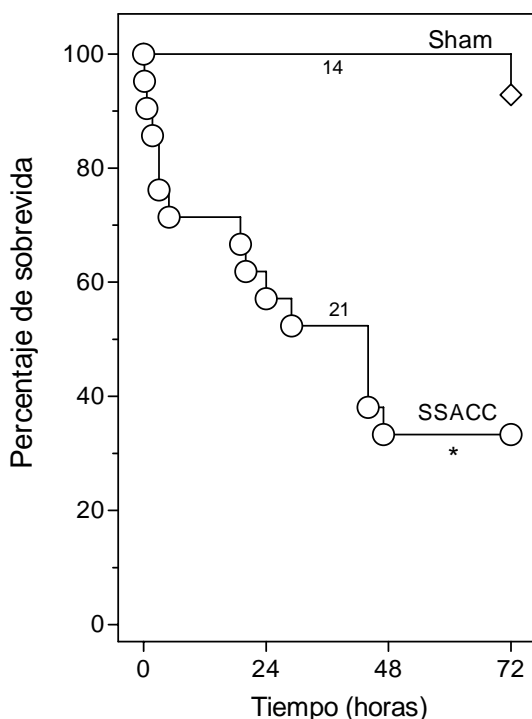
Figura 5. Curvas de sobrevida en ratones del grupo control (sham, ◇) y en ratones sometidos a la SSACC (O). El registro de las muertes fue continuo en las primeras 6 horas después de la segunda cirugía, cada 6 horas durante las primeras 24 h, y después, cada 12 h hasta el fin del experimento (72 h). Los datos son derivados de un número variable de animales al inicio de los experimentos, como se indica en cada curva. Las abscisas representan el tiempo después de la segunda cirugía, la ordenadas el porcentaje de sobrevida. Las curvas fueron calculadas por el método de Kaplan-Meier y comparadas usando la prueba de rangos logarítmicos (*p<0.001).

5.2   Discapacidad neurológica.

Ningún animal del grupo sham mostró anormalidades neurológicas detectables, mientras que los animales del grupo con SSACC mostraron un perfil consistente de alteraciones neurológicas. La figura 6 muestra el puntaje neurológico de los animales control (sham) en comparación con los ratones experimentales sometidos a la SSACC. Las medianas de los índices de discapacidad neurológica para los animales sham fueron de 0 durante todo el periodo de observación. Las medianas del índice neurológico en los ratones con SSACC fueron 8, 10 y 10 a las 24, 48 y 72 horas, respectivamente; los cuales fueron considerablemente mayores (p<0.0001) que los ratones del grupo control.
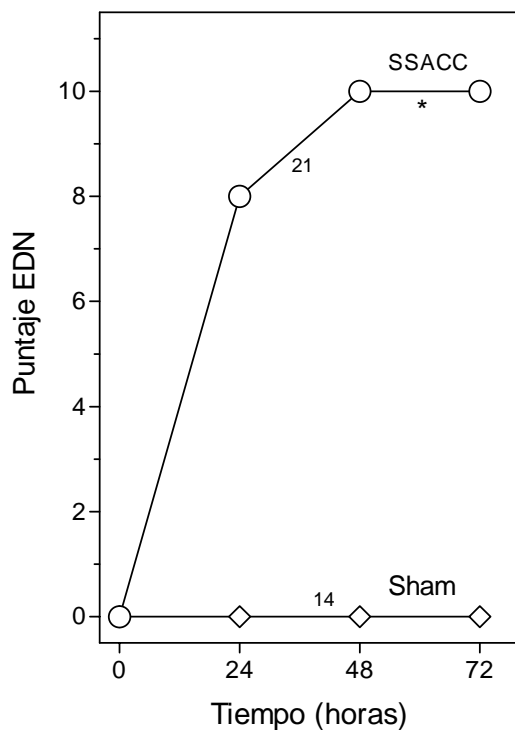
Figura 6. Curvas de los puntajes neurológicos en ratones controles (sham, ◇) y en ratones sometidos a la SSACC (○). El grado de disfunción neurológica fue calificado en 6 pasos progresivos: 0 representa no disfunción neurológica; 2 indica discapacidad mínima; 4 representa disfunción moderada; 6 representa animales más dañados; 8 refiere discapacidad severa; y 10 indica muerte debido a SSACC. Los animales fueron examinados antes de la segunda cirugía y 24, 48 y 72 horas después. La abscisa representa tiempo después de la cirugía, la ordenada, el grado de disfunción neurológica. Los valores indicados son medianas de 14 animales en el grupo sham y 21 animales en el grupo de SSACC al inicio de los experimentos. Se usó la prueba de U de Mann-Whitney para la comparación entre los grupos.

5.3 Efecto de los antioxidantes en estudio sobre la mortalidad y la discapacidad neurológica producida por la SSACC.

La administración de AA (500 mg/kg) y DHLA (100 mg/kg) disminuyó claramente el número de muertes de los animales sometidos a SSACC. Las proporciones de sobrevida a las
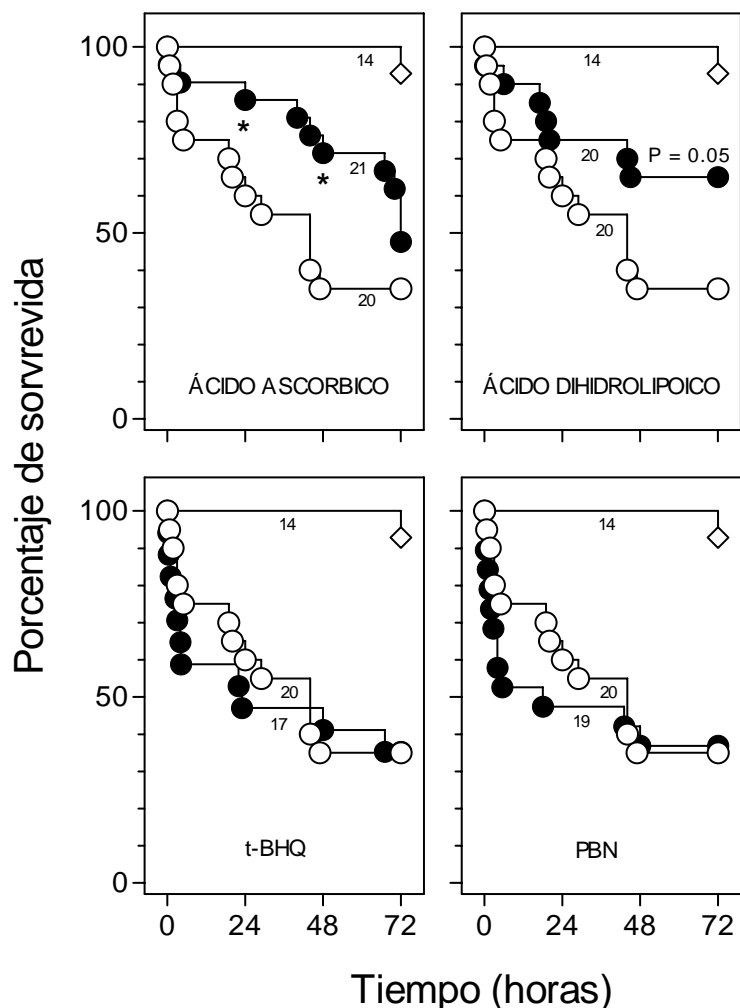
Figura 7. Efecto de los antioxidantes sobre la mortalidad en ratones inducida por la SSACC. Cada panel muestra las curvas de sobrevida para los animales sham ($\diamond$), y tratados con solución salina ($\bigcirc$), y con antioxidantes ($\bullet$). Quince min después de la segunda cirugía, se administró vía i.p. AA (500 mg/kg), DHLA (100 mg/kg), t-BHQ (100 mg/kg), PBN (100 mg/kg) o solución salina. Las muertes fueron anotadas continuamente por un periodo de 6 horas y después cada 12 horas hasta los 3 días. Los datos son derivados de un número variable de animales (n = 14-21) al inicio de los experimentos, como se indica en cada curva. La abscisa representa el tiempo después de la administración; las ordenadas el porcentaje de sobrevida. Las curvas de sobrevida fueron calculadas por el método de Kaplan-Meier y comparadas usando la prueba de rangos logarítmicos (2 colas). Los asteriscos denotan diferencias con respecto al grupo tratado con solución salina (p < 0.05).

24, 48 y 72 h fueron 86, 71 y 48% y 75, 65 y 65% para AA y DHLA, respectivamente. Las proporciones de sobrevida en animales tratados con solución salina fueron 60, 35 y 35% (Fig. 7). La prueba de rangos logarítmicos reveló que las curvas de sobrevida son significativamente diferentes para los animales tratados con AA a las 24 y 48 horas. En el caso de DHLA el incremento en la sobrevida no alcanzó significancia estadística (p = 0.05). En la misma figura se muestra que t-BHQ a la dosis de 100 mg/kg y que PBN a la dosis de 100 mg/kg no aumentaron la proporción de sobrevida de los animales sometidos a SSACC.

La figura 8 muestra los índices de discapacidad neurológica de los animales que recibieron los fármacos en estudio. Los animales tratados con AA, pero especialmente los que recibieron DHLA disminuyeron consistentemente los puntajes neurológicos totales. Las medianas de los puntajes neurológicos obtenidos a las 24, 48 y 72 h con AA (6, 7 y 10) y DHLA (3, 4 y 5) fueron significativamente menores que las medianas de los animales tratados con solución salina (8, 10 y 10). Para ambos fármacos, la comparación reveló diferencias significativas a las 24 y 48 h. En el caso de DHLA, los puntajes también fueron menores a las 72 horas, aunque no fue significativa debido a la variabilidad encontrada. Ni la administración de t-BHQ o PBN no disminuyó los índices neurológicos.
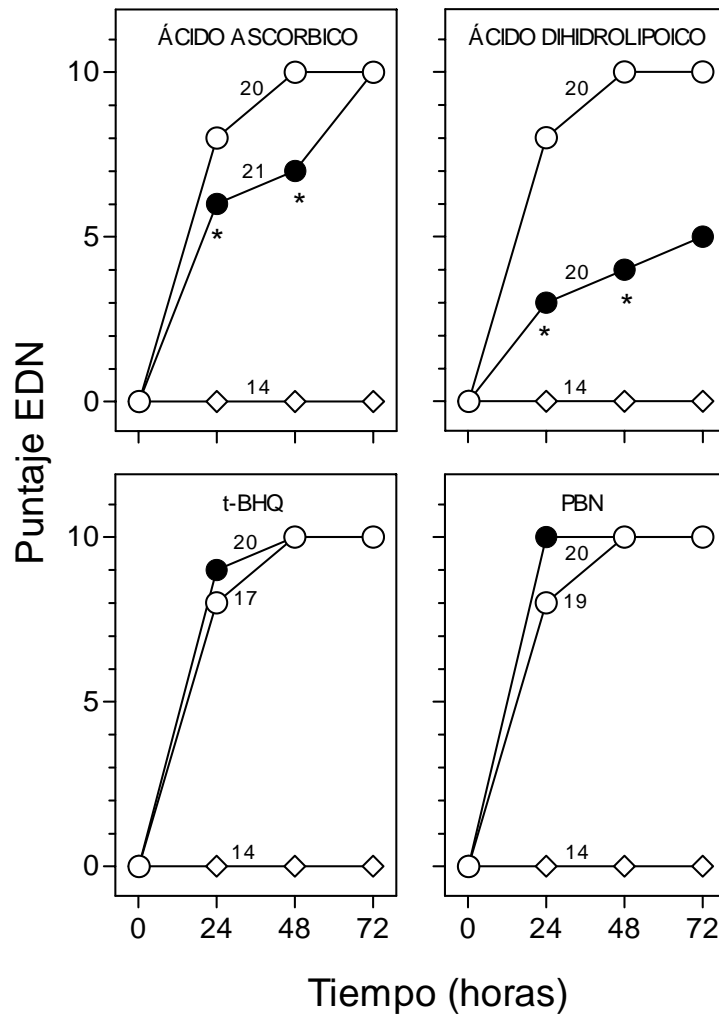
Figura 8. Efecto de los antioxidantes sobre la discapacidad neurológica inducida en ratones por la SSACC. Cada panel muestra las medianas de los índice de discapacidad neurológica para los animales sham (◇), los tratados con solución salina (O), y los tratados con fármacos antioxidantes (●). Quince min después de la segunda cirugía, se administró vía i.p. AA (500 mg/kg), DHLA (100 mg/kg), t-BHQ (100 mg/kg), PBN (100 mg/kg) o solución salina. El grado de daño neurológico fue graduado en 6 pasos progresivos: 0 representa que no existe disfunción neurológica; 2 indica incapacidad mínima; 4 disfunción moderada; 6 representa animales más incapacitados; la categoría 8 refiere discapacidad severa; y 10 indica muerte por SSACC. La abscisa representa el tiempo después de la administración; las ordenadas el grado de disfunción neurológica. Los símbolos corresponden a medianas de los índices neurológicos para 14-21 animales, como se indica en cada curva. Los asteriscos denotan reducción significante (p < 0.05) en los puntajes de discapacidad comparado con el grupo tratado con solución salina (Kruskal-Wallis seguida por la prueba de Dunn).

5.4 Efectos de los fármacos en estudio sobre la hipomotilidad espontánea inducida por la SSACC.

La motilidad de los ratones del grupo control (sham) disminuyó gradualmente con la medición repetida, pero las reducciones sólo fueron estadísticamente significantes a las 72 horas cuando se comparan con el registro anterior a la segunda cirugía (basal) (Fig. 9). Excepto en un punto (48 h), la motilidad de los animales tratados con salina fue significativamente menor que el observado en los sham. La motilidad de los ratones tratados con DHLA consistentemente fue mayor que los registrados para los tratados con solución salina, pero la diferencia solo fue significativa a las 48 horas. No se encontraron diferencias en la motilidad entre los animales que recibieron AA, t-BHQ o PBN y los tratados con solución con salina.
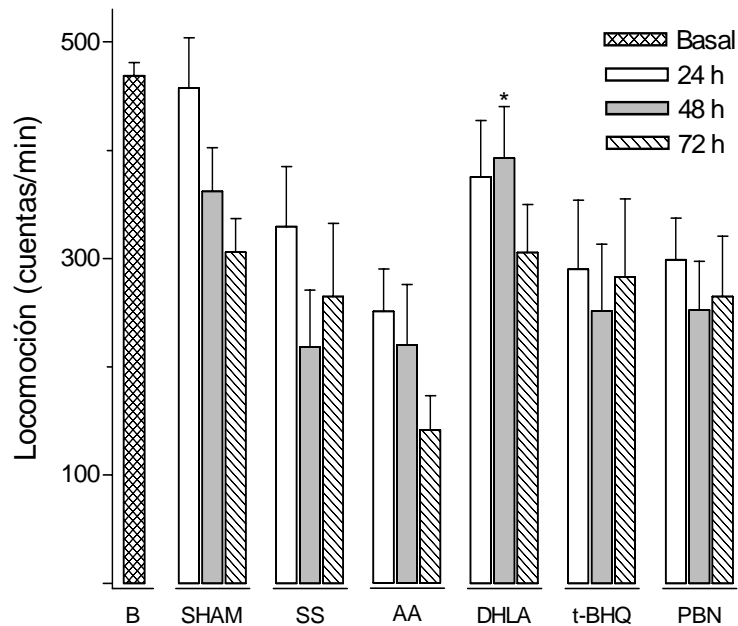
Figura 9. Efecto de los antioxidantes sobre la hipomotilidad en ratones inducida por la SSACC. Las barras cruzadas representan el promedio de la basal (B). Cada grupo de tres barras representa la motilidad a las 24 (vacía), 48 (gris) y 72 (diagonal) horas después de la sección de la carótida contralateral. Quince min después de la segunda cirugía, se administró vía i.p. AA (500 mg/kg), DHLA (100 mg/kg), t-BHQ (100 mg/kg), PBN (100 mg/kg) o solución salina (SS). La motilidad espontánea fue medida por un periodo de un minuto y cada barra representa la media de un número variable de animales (14-21) al inicio del experimento. Líneas verticales indican errores estándar. * $p < 0.05$, comparados con el correspondiente barra de animales tratados con solución salina (ANOVA seguida de la prueba de Dunnett).

5.5 Efectos de los fármacos en estudio sobre la pérdida de peso corporal inducida por la SSACC.

Todos los animales operados perdieron peso (Fig. 10). Los ratones sham perdieron casi 1.7 g en promedio, comparado con su peso preoperatorio. En contraste, los animales tratados con solución salina perdieron una proporción significante de su peso corporal. En este grupo, la diferencia entre el peso prequirúrgico y los pesos a las 24, 48 y 72 horas después de la segunda cirugía fueron 5.4 ± 1.1, 6.5 ± 1.3, y 8.5 ± 1.6, respectivamente. De los antioxidantes probados, el ácido dihidrolipoico disminuyó la pérdida de peso corporal, aunque la diferencia no fue estadísticamente significativa ($p > 0.5$, ANOVA).
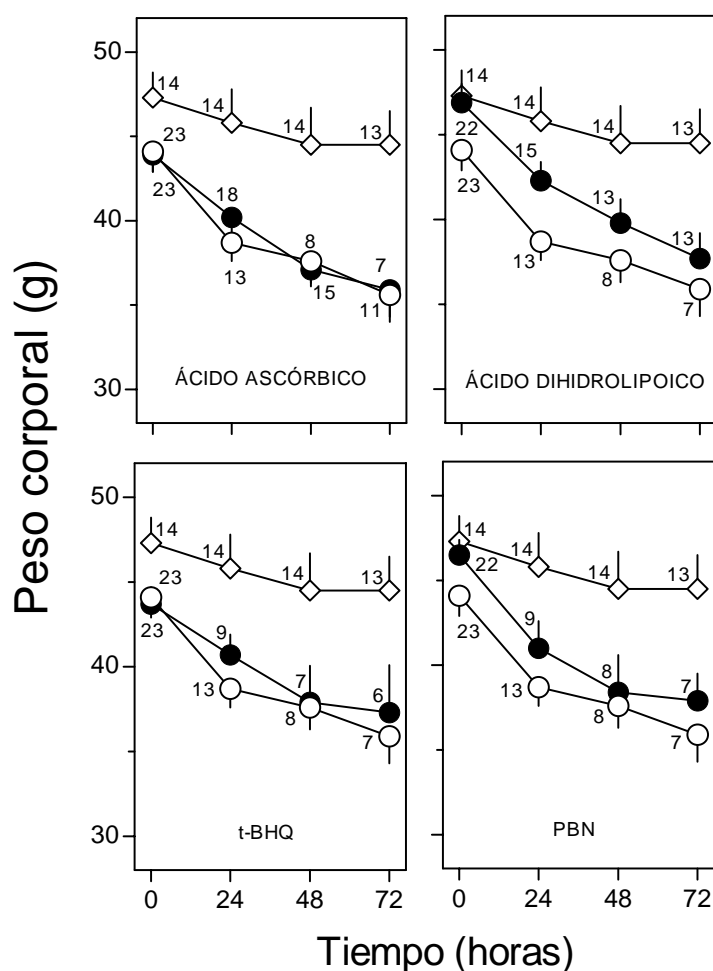
Figura 10. Efecto de los antioxidantes sobre el peso corporal en ratones sometidos a la SSACC. Cada panel muestra las medias de los pesos para los ratones sham ($\diamond$), tratados con salina ($\bigcirc$), y tratados con antioxidantes ($\bullet$). Quince min después de la segunda cirugía, se administró vía i.p. AA (500 mg/kg), DHLA (100 mg/kg), t-BHQ (100 mg/kg), PBN (100 mg/kg) o solución salina. Las abscisas representan tiempo después de la segunda cirugía, las ordenadas el peso corporal en gramos. Cada símbolo representa la media $\pm$ error estándar del número de animales indicados.

IV. DISCUSIÓN

Los resultados más importantes de este estudio demuestran: 1) que la sección secuencial de las arterias carótidas comunes produce una gran variedad de alteraciones neuroconductuales y mortalidad elevada; 2) que la estrategia desarrollada es útil para identificar y cuantificar las alteraciones neurológicas producidas por la SSACC; y 3) que de los antioxidantes probados, el ácido dihidrolipoico es el más eficaz para disminuir la mortalidad y el déficit neurológico producido por la SSACC.

Con relación al primer señalamiento, este estudio revela la presencia de 23 alteraciones neuroconductuales cuya frecuencia y severidad es diferente. Por su naturaleza, estas alteraciones se pueden agrupar en cuatro categorías: a) motoras, que incluyen incoordinación motora, postura lateralizada, postura aplanada, disminución del tono y fuerza muscular, temblor, encorvamiento, flexión de la extremidad anterior, marcha atáxica, desplazamiento en círculo, rotación corporal, sacudidas, convulsiones; b) conductuales, entre las que destacan hipomotilidad, pasividad, irritabilidad, hiperreactividad; c) autonómicas, como incontinencia urinaria, piloerección, dificultad respiratoria; y d) sensoriales, como hipoalgesia e hiperalgesia.

Este resultado es importante, ya que las alteraciones neuroconductuales provocadas por otros procedimientos experimentales de isquemia cerebral son muy limitadas. En el modelo de oclusión unilateral de la arteria cerebral media en ratas, utilizando varias pruebas, se han reportado alteraciones sensorimotoras contralaterales al sitio de lesión, como flexión de la extremidad anterior (van der Staay y cols., 1996), lateralización de la cabeza o el cuerpo, desplazamiento en círculo (Bederson y cols., 1986), falta de respuesta a estímulos, rotación corporal (evidentes con la administración de anfetamina) (Grabowski y cols., 1988; 1991; 1993); también se han reportado déficits cognitivos y conductuales, aunque los reportes son menos consistentes y, en algunos casos, contradictorios (Hunter y cols., 1998; DeVries y cols., 2001); no se reportan alteraciones autonómicas.

Por otro lado, se ha demostrado que en los modelos experimentales con daño bilateral extenso del cerebro anterior (hipocampo, estriado y corteza) se presentan alteraciones sensorimotoras y, de manera más frecuente, alteraciones cognitivas y de memoria (Block, 1999). En ratas y jerbos con isquemia global transitoria, usando diferentes pruebas, se ha reportado que se altera la actividad locomotora espontánea (Chandler y cols., 1985); Gerhardt y Boast, 1988; Wang y Corbett, 1990), la capacidad de respuesta (Ginsberg y Busto, 1989), la posición corporal, el reflejo de enderezamiento (Ginsberg y Busto, 1989;

Pulsinelli y Brierley, 1979; Bederson, 1986), la colocación visual de la pata (Schallert y cols., 2000), el balance sobre el rotarod (Combs y D'Alecy, 1987; Gionet y cols., 1991), aprendizaje y memoria (Nunn y Hodges, 1994; Block, 1999); asimismo, que se producen convulsiones (Blomqvist y cols., 1984; Tsuchiya y cols., 2003).

El problema principal de la evaluación funcional en los modelos tradicionales de isquemia cerebral, especialmente después de la isquemia transitoria del cerebro anterior, es que expresan un perfil limitado de alteraciones y conductas con recuperación espontánea rápida del déficit sensorial y motor (Block, 1999). Capdeville y colaboradores (1986), en ratas con oclusión permanente de las arterias vertebrales y oclusión por 30 min de las carótidas comunes, encontraron que varias alteraciones (reflejo de enderezamiento, equilibrio, reflejo de flexión, motilidad espontánea, fuerza y coordinación muscular) alcanzaron su pico máximo en una hora, y estaban presentes a las 3 horas; sin embargo, desaparecieron 24 h después de la isquemia. Combs y D'Alecy (1987) y Gionet (1991) en el modelo de oclusión de 4 vasos con 20 min de isquemia, reportaron daño en la fuerza y coordinación muscular 24 h posteriores a la isquemia, pero no después.

Como se puede apreciar, para propósitos de evaluación funcional es necesario tener un modelo que provoque daño orgánico y alteraciones neuroconductuales severas y persistentes. Se sabe que el modelo de oclusión secuencial de las arterias carótidas comunes provoca daño bilateral extenso en hipocampo, estriado y corteza (Rodriguez y cols., 2000a); quizá esto explique el rango tan amplio de manifestaciones incluyendo autonómicas, sensoriales, motoras y conductuales que son propias de los modelos de isquemia que involucran daño a estas regiones. Es importante mencionar que la mayor parte de estas alteraciones no se recuperan y que algunas de ellas aumentan progresivamente su severidad (debilidad muscular y encorvamiento) (Rodriguez y cols., 2005). Los resultados de la revisión bibliográfica que llevamos a cabo revelaron que no hay estudios que describan los efectos funcionales de la isquemia bilateral del cerebro anterior en ratones, y sólo algunos autores han intentado describir el perfil funcional completo después de la isquemia global en ratas (Capdeville y cols., 1986). Por el contrario, se ha reportado una caracterización detallada de los déficits sensorimotores en ratones y ratas después de la isquemia cerebral focal (Hunter y cols., 2000; Chen y cols., 2001).

Por otro lado, los ratones con SSACC mostraron una mortalidad elevada (64%) durante el periodo de observación (72h). Recientemente, se ha estado expresando que el mejor indicador del daño isquémico es una combinación entre mortalidad y alteraciones

funcionales (Hunter y cols., 1998). Sin embargo, pocos estudios (Floyd, 1990; Panigrahi y cols., 1996; Li y cols., 2001; Huang y cols., 2001) han tomado en cuenta a la mortalidad como indicador de daño y neuroprotección. La mayor parte de investigadores no toman en cuenta, o al menos no reportan, a la mortalidad como un indicador en animales isquémicos (Grotta, 1996; Aspey y cols., 1998; Modo y cols., 2000). Por estas razones, es un parámetro poco explorado en esta área.

Con relación al segundo señalamiento, cabe mencionar el interés actual en las evaluaciones funcionales. Como se ha descrito antes, se ha cuestionado fuertemente el uso de modelos que se empeñan en determinar al volumen de la lesión como un indicador único de daño y neuroprotección farmacológica (Corbett y Nurse, 1998; de Keyser y cols., 1999). Varios autores han expresado que la mortalidad y el daño funcional pueden ser indicadores más útiles (Hunter y cols., 1998; Block, 1999). Se ha considerado que mediciones de la capacidad funcional en animales pueden generar información muy valiosa, útil para determinar la presencia, severidad y evolución temporal del déficit, y de las consecuencias de las intervenciones farmacológicas (Rodriguez y cols., 2005).

Cabe mencionar que la estrategia aquí descrita para identificar las alteraciones autonómicas, sensorimotoras y conductuales en el modelo de SSACC se basan en el método observacional validado por Irwin (1968). Este procedimiento tiene la ventaja de ser relativamente sencillo, barato y, más importante aun, con un observador bien entrenado produce resultados consistentes y reproducibles. Irwin lo diseñó para el cernimiento de fármacos con actividad sobre el sistema nervioso central (Warburton, 2002). Cabe destacar que, en analogía a la situación clínica, involucra una fase inicial de observación y, posteriormente, una fase de exploración. Como se puede apreciar, el diseño de este trabajo tiene como fundamento una evaluación global, sistemática, simplificada y rápida. Con esta estrategia obtuvimos información sobre el estado de alerta, locomoción, interacción social, postura, coordinación, tono muscular, reflejos, así como de la función autonómica. Además nos da información sobre conductas como motivación, discriminación, y adquisición o extinción de ciertos patrones conductuales. Irwin consideró que, con este sistema de evaluación, una mayor precisión no tiene importancia práctica, mientras que en el afán de objetividad y precisión instrumental se puede perder información valiosa (Warburton, 2002).

Ante la gran variedad de alteraciones neurológicas presentes en ratones con SSACC, y para tener un indicador práctico de daño funcional, fue necesario desarrollar una escala que determinara el nivel de discapacidad neurológica en función del tiempo. Basados en una

escala clínica desarrollada por Kurtzke (1983), que de manera progresiva gradúa la discapacidad en pacientes con esclerosis múltiple, desarrollamos una escala de discapacidad neurológica (EDN). La escala comprende 6 principales grados o pasos que aumentan progresivamente. Inicia en 0 para animales sin alteraciones aparentes, y se va extendiendo a 2 para discapacidad leve, 4 para discapacidad moderada, 6 para animales más discapacitados pero con posibilidad de movimiento, 8 para animales inmóviles con dificultad respiratoria, y 10 para muerte debida a la SSACC. Puesto que cada nivel representa la suma de los daños neurológicos, permite una graduación del daño de la función cerebral independientemente de las estructuras involucradas.

Con la aplicación de esta escala, los ratones con SSACC mostraron puntajes de discapacidad consistentemente altos a las 24 h (Fig. 6), y no mostraron evidencia de recuperación a las 48 y 72 h; al contrario, los datos indican algún empeoramiento en función del tiempo ($p > 0.05$), y la necesidad, en futuras investigaciones, de extender el periodo más allá de 72 h. Cabe destacar que, excepto para ptosis, debido a la manipulación de los nervios por el procedimiento quirúrgico (presente en grupo sham y grupo con SSACC), el procedimiento no produce cambios en los animales sham. La escala permite la medición cuantitativa del grado del déficit neurológico antes (basal) y después de la isquemia cerebral y, al mismo tiempo, permite hacer comparaciones periódicas dentro o entre los grupos de animales. El número relativamente alto de disfunción neuroconductual y la marcada diferencia entre los animales del grupo sham y del grupo con SSACC ofrecen la posibilidad de probar el efecto de los tratamientos y monitorear la evolución de los déficits sobre el tiempo en los grupos de animales. Esta escala ha permitido detectar que el dexrazoxano tiene propiedades neuroprotectoras excepcionales (Rodriguez y cols., 2003b).

Para otros modelos se han empleado varias escalas e índices neurológicos que miden la severidad del daño funcional (Nunn y Hodges, 1994; Corbett y Nurse, 1998; Block, 1999; DeVries y cols., 2001). Sin embargo, estas escalas están diseñadas a evaluar sólo el déficit sensorial y motor en modelos de isquemia focal en ratas (Bederson, 1986; Menzies, 1992; Chen y cols., 2001). A la fecha, ninguna técnica ha recibido aceptación universal y mucho de lo que se ha hecho para evaluar los efectos de la isquemia global y sus posibles tratamientos se basa en diferentes paradigmas. Con frecuencia, además del tamaño del infarto, sólo se incluye un parámetro funcional (por ejemplo, actividad locomotora).

Considerando: 1) que la efectividad de las manipulaciones farmacológicas pueden ser determinadas sólo si la severidad del déficit pueden ser identificado y cuantificado en dos o

más puntos en el tiempo, y 2) la necesidad de resultados con mediciones de neuroprotección terapéuticamente relevantes, se determinó el efecto neuroprotector de los antioxidantes.

Finalmente, la contribución más importante de este trabajo es la demostración de que, el ácido dihidrolipoico es altamente efectivo para reducir la mortalidad y la discapacidad neurológica producida por la isquemia cerebral (Fig. 6, 7 y 8). Estos efectos representan una propiedad importante del DHLA, ya que otros neuroprotectores potenciales, tales como deferoxamina, dizocilpina, y nimodipina, no reducen la mortalidad ni disminuyen consistentemente el déficit neurológico que ocurre después de la SSACC (Rodriguez y cols., 2003b). Los hallazgos con DHLA, y con AA, son compatibles con otras investigaciones que señalan que estos fármacos disminuyen el daño cerebral isquémico. También se ha reportado que el DHLA reduce la mortalidad inducida por la oclusión bilateral de las arterias carótidas más hipotensión en ratas (Panigrahi y cols., 1996).

Como se ha mencionado previamente, un gran número de estudios han demostrado la eficacia de diferentes antioxidantes, incluyendo AA (Stamford y cols., 1999; Henry y Chandy, 1998), DHLA (Prehn y cols., 1992; Backhaus y cols., 1992; Panigrahi y cols., 1996; Cao y Phillis, 1995; Wolz y Krieglstein, 1996) y PBN (Phillis y Clough-Helfman, 1990; Cao y Phillis, 1994; Zhao y cols., 1994;Yang y cols., 2000; Li y cols., 2001), y t-BHQ (Murphy y cols., 1991) para reducir el daño cerebral isquémico en modelos de isquemia global/focal en jerbos, así como en modelos de isquemia focal permanente y transitoria en ratas y ratones. Sin embargo, aunque estudios recientes de neuroprotección en los modelos tradicionales de isquemia cerebral han puesto mayor interés en la evaluación funcional, las pruebas elegidas en los estudios preclínicos no reflejan a las usadas en los ensayos clínicos. Pocos estudios han examinado los efectos de los antioxidantes sobre la mortalidad (Floyd, 1990; Panigrahi y cols., 1996; Li y cols., 2001) y/o el resultado funcional después de la isquemia cerebral (Cao y Phillis, 1995; Wolz y Krieglstein, 1996; Yang y cols., 2000), y éstas usan solo un parámetro (por ejemplo, actividad locomotora).

Por otro lado, los mecanismos responsables del efecto neuroprotector de AA y DHLA en nuestro modelo experimental de isquemia no fueron revisados en este estudio, pero pueden atribuirse a su acción antioxidante, como ha sido el caso de su habilidad para limitar el tamaño del infarto en animales isquémicos. El AA es un potente atrapador de radicales libres, tiene la capacidad de donar dos electrones y así autooxidarse a ácido dehidroascórbico (Rice, 2000); también recicla a otros antioxidantes tales como la vitamina

E, lo cual interrumpe la lipoperoxidación de las membranas celulares (Seregi y cols., 1978). Cabe mencionar que el AA no cruza fácilmente la barrera hematoencefálica en estado normal, así que la neuroprotección observada en este y en otros estudios podría ser explicado por entrar al cerebro por mecanismos no conocidos o vía una barrera hematoencefálica temporalmente abierta (Preston y cols., 1993). El ácido dihidrolipoico es un antioxidante que cruza la barrera hematoencefálica. Para este fármaco se han demostrado cuatro propiedades antioxidantes: es un quelante de metales de transición (Scott y cols., 1994; Suh y cols., 2004); atrapa especies reactivas de oxígeno (Kagan y cols., 1992); regenera antioxidantes endógenos, incluyendo al glutation y a las vitaminas E y C (Packer y cols., 1995), y puede reparar el daño oxidativo (Biewenga y cols., 1997). Además, modula las actividades de factores de transcripción, especialmente la de NF-κB (Packer y cols., 1997).

Los resultados negativos con PBN contrastan con los resultados de estudios previos, los que indican que este fármaco reduce el volumen del infarto en ratas y jerbos sujetos a isquemia focal o global (Cao y Phillis, 1994). PBN también es un atrapador potente de radicales libres. Este reacciona con los radicales con base de oxígeno y carbono para formar aductos estables (Yang y cols, 2000; Kotake, 1999). Las discrepancias entre nuestros hallazgos con PBN y las reportadas por otros autores pueden tener varias explicaciones. La primera, y más importante, son las cuestiones metodológicas concernientes al modelo experimental y los criterios para medir los efectos de la isquemia cerebral y el grado de neuroprotección. El modelo de la SSACC es, esencialmente, un modelo de isquemia global incompleta con una diferencia importante. En la mayoría de los modelos de isquemia del cerebro anterior, la oclusión de las arterias carótidas es transitoria, y tanto el inicio de la isquemia como su reversión subsecuente son abruptos (Ginsberg y Busto, 1989). En nuestro modelo, la sección de la carótida común derecha, en animales previamente sometidos a una reducción del suministro sanguíneo por oclusión previa de la arteria carótida común izquierda, produce isquemia severa del cerebro anterior. Postulamos que este modelo puede ser relevante para el evento cerebrovascular agudo, ya que implica provocar isquemia aguda en animales ya sometidos a hipoperfusión crónica, como ocurre en humanos. Además, usa la sobrevida y una escala de discapacidad neurológica (como en la clínica), en lugar del volumen del infarto, como indicadores para determinar los efectos de la isquemia cerebral y el grado de neuroprotección farmacológica. Otra consideración importante, es que los estudios previos con PBN fueron conducidos en animales jóvenes o

adultos jóvenes. Las diferencias en susceptibilidad a la isquemia cerebral entre los jóvenes y viejos han sido claramente establecidos (Yager y cols., 1996; Fuentes-Vargas y cols., 2002). También es posible que los tratamientos eficaces en animales jóvenes no sean igualmente eficaces en animales viejos.

La falla de t-BHQ y PBN en nuestro modelo experimental de isquemia cerebral apunta la posibilidad de que la eficacia de AA y de DHLA esté relacionada a su capacidad de interrumpir mecanismos de estrés oxidativo a diferentes puntos en la isquemia cerebral. Claramente, existe una variedad de mecanismos adicionales que podrían explicar las propiedades neuroprotectoras de estos fármacos (Clemens, 2000; Moini y cols., 2002; Dewar, 1999; Arivazhagan y Panneerselvam, 2000; McCarty, 2001), aun cuando nuestros resultados no revelan la naturaleza de tales mecanismos.

Nuestra estrategia para producir isquemia cerebral y para evaluar el resultado funcional es sencilla y genera resultados consistentes. Sin embargo, tiene algunas desventajas. Nuestro procedimiento de evaluación no es suficientemente específico para registrar cambios pequeños en los individuos, es laborioso, costoso en tiempo, requiere un tamaño de muestra grande de animales, condiciones experimentales apropiadas, y tiempo para entrenar a una persona que identifique satisfactoriamente tanto la conducta normal del ratón como las alteraciones neuroconductuales. Su principal ventaja podría ser su capacidad para discriminar neuroprotección relevante (Rodriguez y cols., 2003b; Santiago-Mejia y cols., 2005).

Finalmente, este trabajo confirma que la SSACC produce un patrón de mortalidad y un perfil de daño funcional consistentes y que nuestra estrategia metodológica permite determinar la severidad del déficit funcional. Asimismo, establece su utilidad para identificar fármacos neuroprotectores.

En resumen, la administración de AA y de DHLA incrementa la sobrevida y disminuye las alteraciones neurológicas en ratones sometidos a la SSACC. Estos resultados indican que estos fármacos, principalmente DHLA, pueden ser eficaces en pacientes con isquemia cerebral aguda. Nuestra estrategia para evaluar la eficacia neuroprotectora es suficientemente sensible para detectar diferencias entre miembros de un mismo grupo de fármacos. Estos resultados confirman la validez y utilidad de nuestro modelo de isquemia cerebral aguda.

VII.   CONCLUSIONES

1. La sección secuencial de las arterias carótidas comunes produce una gran variedad de alteraciones neuroconductuales cuantificables y un patrón consistente de mortalidad.

2. La estrategia para identificar y cuantificar las alteraciones neuroconductuales es útil en la búsqueda de neuroprotectores.

3. De los antioxidantes probados, el ácido dihidrolipoico es el más eficaz para disminuir la mortalidad y el déficit neurológico producidos por la isquemia cerebral.

4. Nuestra estrategia para evaluar la eficacia neuroprotectora es suficientemente sensible para detectar diferencias entre miembros de un mismo grupo de fármacos.

5. Estos resultados confirman la validez y utilidad de nuestro modelo de isquemia cerebral.

6. Estos hallazgos apoyan la idea de que estos fármacos, principalmente DHLA, puedan tener eficacia neuroprotectora en pacientes con isquemia cerebral aguda.

VIII.   REFERENCIAS BIBLIOGRÁFICAS

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A simplified procedure for the quantitative measurement of neurological deficits after forebrain ischemia in mice

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Abstract

This study describes a comprehensive method to assess neurological deficits after brain ischemia produced by sequential common carotid artery sectioning (SCAS) in aged mice, and a scale to determine the degree of functional incapacity of ischemic animals. The method involves an initial phase of undisturbed observation and a later manipulative phase during which each animal is subjected to a sequence of very simple manipulations. Sham-operated animals demonstrated 96% survival throughout the study period (72 h), whereas the 24, 48 and 72 h survival rates of SCAS-mice were 48, 38 and 36%, respectively. In the surviving SCAS-mice, we detected a total of 23 neurological alterations throughout the observation period (72 h); the most frequent alterations were: motor incoordination, abnormal body position, hypomobility, decreased body tone and muscular strength, tremor, hunched back, passivity, forelimb flexion and ataxic gait. Based on these alterations, we used a global scale that comprises 10 progressive grades beyond 0 (normal), extending to status 10 (death due to SCAS), with higher scores indicating greater deficit. The median neurological scores for sham-operated animals were 1.36, 1.48 and 1.32 at 24, 48 and 72 h, respectively, whereas total neurological scores in SCAS-mice of 6.1, 6.8 and 7.4, at 24, 48 and 72 h, respectively, were substantially greater than those observed in sham-operated animals. The simplicity of the procedure, herein described, to measure the functional neurological condition of ischemic animals, and the remarkable level of functional impairment produced by SCAS offer the possiblity to test the efficacy of putative stroke therapies and to monitor progress of deficits over time in groups of animals.
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Keywords: Brain ischemia; Stroke; Mice; Models; Neurological deficits; Scale

1. Introduction

The measurement of neurobehavioral disabilities after experimental brain ischemia has become an increasingly important research activity as clinical studies have shown that a large number and variety of compounds highly effective in reducing infarct volume in animal stroke models (Mohamed et al., 1985; Park et al., 1988; Halliwell, 1989; Read et al., 1999; Green et al., 2003) have all failed in human trails of acute ischemic stroke (Hossmann, 1997; Corbett and Nurse, 1998; de Keyser et al., 1999; Green et al., 2003; Wahlgren and Ahmed, 2004).

Since brain injury after stroke in humans is commonly associated with impaired sensorimotor ability, reduced cognitive function and change in affect (Gavrilescu and Kase, 1995; Patel et al., 2003), many laboratories have focused their research on assessing cognitive and behavioral correlates of histologically determined stroke damage in animal models. It is considered that reliable measurements of functional capacity in animals can provide meaningful data to determine presence, severity and time course of impairments, and the consequences of pharmacological interventions. One major problem of functional output assessment is that traditional models of experimental stroke display a somewhat limited behavioral profile, and spontaneous recovery of sensorimotor deficits commonly occurs rapidly, especially after global cerebral ischemia (Block, 1999). In addition, in most cases

it is necessary to employ a variety of different tasks to examine the alterations that occur in rodents after an experimental stroke. Today, no single measurement technique has yet proven to be universally acceptable and most recently proposed protocols (Hunter et al., 2000) lack information regarding their capacity to detect pharmacological neuroprotection.

Previous studies from this laboratory have shown that sequential common carotid artery sectioning (SCAS) produces a reproducible pattern of mortality, and significant morphological and behavioral changes in mice (Rodriguez et al., 2000). The present study describes a comprehensive method to assess neurological deficits after brain ischemia produced by SCAS, and the strategy used to quantify the degree of functional incapacity in ischemic animals; strategy that has already been used to evaluate pharmacological neuroprotection (Rodriguez et al., 2003).

2. Methods

2.1. Animals

This study was performed in male CFW, aged 40–60 weeks mice, weighing 40–50 g at the beginning of the experiments. Animals were obtained from our breeding facilities and housed 3–5 per cage (same litter) in a temperature-controlled room ($22 \pm 2\,^\circ$C, relative humidity $55 \pm 3\%$), with an automatically timed cycle of 12-h light:12-h dark (lights on 08:00–20:00). Food (Purina Chow, St. Louis, MO) and water were available ad libitum. Mice were allowed to acclimate to these environmental conditions for at least 1 week prior to experimentation. Twelve hours before experiments, food was withheld but free access to water was maintained. Surgery and testing were performed between 09:00 and 14:00 h during the light period. All experiments were carried out under the provisions of the Declaration of Helsinki, and adhered to the National Health Ministry guidelines for the use of laboratory animals.

2.2. Surgery

Brain ischemia was produced as described previously (Rodriguez et al., 2000). Briefly, light anesthesia was induced with ether delivered through a facemask, and the left common carotid artery was exposed through a midline neck incision and separated from the associated vagus and sympathetic nerves. The artery was sectioned between ligatures and the incision was closed with surgical thread. After surgery, animals were kept in a recovery room for about 1 h under heat lamps to maintain body temperature. Once animals regained complete consciousness, they were moved to the environmentally controlled room. Thirty-two days later, mice were clinically evaluated using the procedure described below. Mice showing any degree of neurobehavioral deficit were excluded from the following

experiments. Animals were randomly assigned to three different groups: experimental mice undergoing anesthesia and sectioning of the right common carotid artery ($n = 50$), sham-operated mice undergoing anesthesia and full surgical procedures, except for artery ligation and sectioning ($n = 25$) and mice not subjected to anesthesia and surgery ($n = 25$).

2.3. Survival

Survival was scored continuously over the first 6 h after the second surgery and then every 6 h during the first 24 h; thereafter, every 12 h up to the end of the experiment (72 h).

2.4. Clinical examination

The procedure used is an adaptation of that validated by Irwin (1968). It involves an initial phase of undisturbed observation and a later manipulative phase during which each animal is subjected to a sequence of manipulations, first starting with the least provoking stimuli (Irwin, 1968).

Mice were individually tested in a dimly lit, quiet room. They were removed from their home cages and placed in the middle of a three-sided wall tabletop (wooden, 60 cm × 60 cm × 20 cm). Neurobehavioral examination, as described below, was carried out just before applying anesthesia for the second surgery, and at 24, 48 and 72 h thereafter. The examiner had no knowledge of the procedure that the mouse had undergone.

Clinical examination was initiated after simultaneously placing on the tabletop the mouse to be evaluated and a mouse, ink marked, with equivalent characteristics, but not belonging to the three described groups (external control, that was substituted with another before each evaluation). As noted later, we registered along the 72 h period of observation the presence of the following 11 alterations during the first phase of the procedure:

- *Hypomobility*. Lower spontaneous mobility of the experimental mouse as compared with that of the external control when placed (30 s) on the top of the table. Scored 1, 2 or 3 in terms of locomotion, and speed and vigor of movements.
- *Lateralized posture*. Abnormal body position characterized by persistent tendency to recline sideward.
- *Flattened posture*. Abnormal body position characterized by slow movements and dragging the body along the tabletop.
- *Hunched back*. Persistent presence of a crouched posture.
- *Piloerection*. Persistent rise of the back hair.
- *Ataxic gait*. Tendency to sway, rock, or lurch to the side as the animal proceeds forward.
- *Circling*. Spontaneous or forced (gently pushing with one finger) walking consistently to one side.
- *Tremors*. Presence of fine, repetitive, oscillatory movements observed during movement.
- *Twitches*. Abrupt body jerks.

- *Convulsions*. Repetitive twitches followed by extension of the hind limbs.
- *Respiratory distress*. Presence of increased, irregular, respiratory movements accompanied by breathing sounds.

The second phase of the procedure, which included a sequence of manipulations, detected the presence of 12 abnormal behavioral elements:

- *Passivity*. Characterized by decreased behavioral response (struggle and escape) when the animals are covered with the hand to restrain movement.
- *Hyperreactivity*. Characterized by exaggerated behavioral response (struggle and escape) when animals are covered with the hand to restrain movement.
- *Irritability*. Characterized by aggressive posture and biting behavior exhibited by the animals when covered with a hand to restrain movement.
- *Ptosis*. Closure or dropping of the upper eyelids.
- *Urination*. Excessive amount of urine on the animal's body and/or the tabletop.
- *Decreased body tone*. Characterized by relatively less resistance to compression or flaccidity of the abdominal muscles, determined by a gentle compression of the sides of the animal between the lower thorax and pelvis using the thumb and index finger.

- *Forelimb flexion*. Failure to extend one forepaw fully when the animal is held by the tail to a height of 10 cm and slowly lowered to observe symmetry in the outstretching of both forelimbs while the mouse reached the wire-mesh.
- *Decreased muscle strength*. Characterized by decreased resistance when the animal is placed on the grid and gently drawn backwards by the tail.
- *Body rotation*. Rolling along the long axis of its body when the animal is held by the tail.
- *Motor incoordination*. Characterized by decreased capacity to move and remain for at least 10 s on the inclined plane (45 °C) and/or to grasp the cord (30 cm above the tabletop) with limbs and tail, and to remain there for at least 10 s. Scored 1, 2 or 3 in terms of persistence, and vigor and coordination of movements.
- *Hypoalgesia*. Lack of behavioral response when an arterial claw is placed at 1 cm from the base of the animal's tail.
- *Hyperalgesia*. Exaggerated behavioral response (disproportional vocalization and biting) when an arterial claw is placed at 1 cm from the base of the animal's tail.

Except for hypomobility and motor incoordination, the occurrence of any of these alterations (phase 1 and 2) was scored with 0 or 1 (0 = not present, 1 = present). Using this strategy the neurological examination of each animal was performed in 3–4 min.
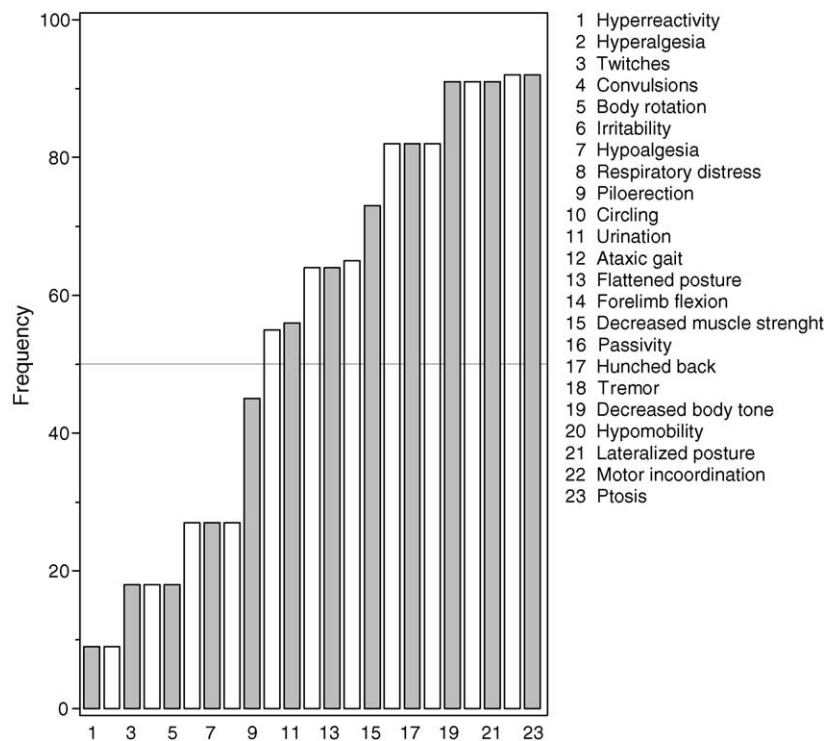
Fig. 1. Frequency of neurobehavioral alterations detected along the 72 h period of observation in mice subjected to sequential carotid artery sectioning. The clinical examination involved an initial phase of undisturbed observation and a later manipulative phase during which each animal was subjected to a sequence of manipulations, first starting with the least provoking stimuli. Animals were examined before and at 24, 48 and 72 h after the second surgery. *n* = 50 at the beginning of the experiment.

2.5. Neurological disability status scale (NDSS)

Based on the results of the clinical examination, showing that SCAS produces a wide spectrum of neurobehavioral alterations, and due to the low frequency of some of them, we decided to take into account only the 14 most frequent and representative alterations induced by SCAS (Fig. 1) to depict severity and progression of the neurological functional impairment after brain ischemia. As shown in Table 1, the neurological disability status scale (NDSS) has 10 progressive steps beyond 0 (normal), extending to status 10 (death due to SCAS); therefore, the higher the number the greater is the neurological dysfunction. Accordingly, the six major steps indicate: zero for normal (no neurological dysfunction, and two represents slight decrease in mobility and the presence of passivity. Category 4 represents moderate neurological dysfunction and includes additional alterations, such as moderate hypomobility, flattened posture, lateralized posture, hunched back, ataxic gait, decreased body tone and muscular strength and slight motor incoordination. Category 6 corresponds to more handicapped animals but still able to walk, with more marked hypomobility, circling, tremor, jerks and/or convulsions, forelimb flexion and moderate motor incoordination. Category 8 corresponds to respiratory distress, and total incapacity to move/coordinate. Status 10 refers death due to SCAS. In all cases, where criteria for the precise grade were not met, the nearest appropriate number was utilized: 1, 3, 5, 7 and 9.

2.6. Data analysis

The survival curves were calculated by the Kaplan–Meier method and compared using the log–rank test. Differences between total neurological scores at the different periods of time, as compared to their respective data for sham-operated animals, were calculated by the Mann–Whitney U-test. Analysis was done using GraphPad Prism Version 3.02 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Frequency of neurobehavioral alterations produced by SCAS

Except for ptosis (90%), none of the sham-operated animals showed neurobehavioral abnormalities, whereas in the surviving SCAS mice a large number of alterations were detected throughout the observation period (24–72 h). Fig. 1 shows the relative frequency of neurobehavioral consequences of SCAS. In addition, hypermobility, stereotypy, increased muscular tone, catatonia, diarrhea were noted in some animals (<5%). Motor incoordination, lateralized posture, hypomobility, decreased body tone, tremor, hunched back, passivity, decreased muscle strength, forelimb flexion, flattened posture and ataxic gait were, in that order, the most consistent alterations produced by SCAS.

3.2. Mortality produced by SCAS

No deaths were observed in control animals (no anesthesia no surgery) and, except for one case (1/25), no deaths were observed in sham-operated mice. Therefore, sham-operated animals demonstrated 96% survival throughout the study period of 72 h. In the SCAS group, 10% of animals died within the first 15 min after the second surgery, and the number of deaths progressively increased to 18% at 60 min, 52% at 24 h, 62% at 48 h and 64% at 72 h. Fig. 2A compares Kaplan–Meier survival curves for sham-operated animals and for mice subjected to SCAS. The 24, 48 and 72 h survival rates of SCAS-mice were 48, 38 and 36%, respectively. The results of the log–rank test indicated highly statistically significant difference between these two curves ($p < 0.0001$).

3.3. Neurological impairment produced by SCAS

Fig. 2B shows the detailed neurological scoring of sham-operated animals in comparison with mice subjected to SCAS. The median NDSS scores for sham-operated animals, mainly due to ptosis, were 1.36 (range, 0.6–2.1), 1.48 (range, 0.7–2.2) and 1.32 (range, 0.6–2.0) at 24, 48 and 72 h, respectively. Differences among these three values were not statistically significant ($p > 0.05$). The median neurological scores of SCAS-mice: 6.1 (range, 4.9–7.3), 6.8 (range, 5.7–7.9) and 7.4 (range, 6.3–8.4), at 24, 48 and 72 h, respectively, were considerably higher ($p < 0.0001$) than those of sham-operated mice. The increasing tendency of these values did not reach statistical significance ($p > 0.05$).

Table 1
Neurological disability status scale

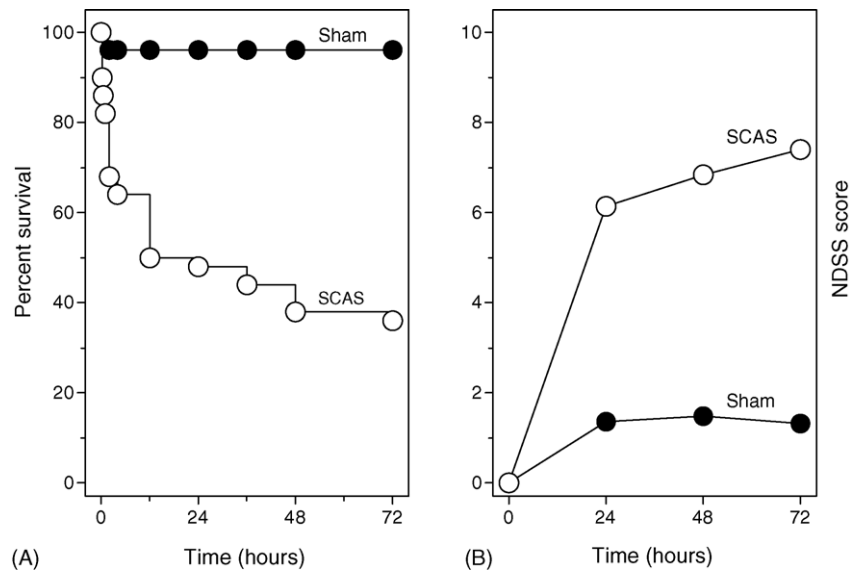| Degree of deficit	Neurobehavioral alterations
0	None
2	Hypomobility (slight)
	Passivity
4	Hypomobility (moderate)
	Flattened posture
	Lateralized posture
	Hunched back
	Ataxic gait
	Piloerection
	Decreased body tone
	Decreased muscular strength
	Motor incoordination (slight)
6	Circling
	Tremor/twitches/convulsions
	Forelimb flexion
	Motor incoordination (moderate)
8	Hypomobility (severe)
	Motor incoordination (severe)
	Respiratory distress
10	Death (due to SCAS)

Fig. 2. Survival curves (panel A) and neurological scores (panel B) in sham-operated controls (●) and mice subjected to SCAS (○). In panel A, deaths were scored continuously over the first 6 h after the second surgery and then every 6 h during the first 24 h. Thereafter, every 12 h up to the end of the experiment (72 h). The abscissa represents time after surgery, the ordinate, percent survival. The survival curves were calculated by the Kaplan–Meier method and compared using log–rank test ($p < 0.0001$). In panel B, the degree of neurological dysfunction was rated on six mayor progressive steps: 0 represents no neurological dysfunction; 2 indicates minimal disability; 4 represents moderate dysfunction; 6 represents more handicapped animals; 8 refers severe disability; 10 indicates death due to SCAS. Animals were scored at 24, 48 and 72 h post-surgery. The abscissa represents time after surgery, the ordinate, grade of neurological dysfunction. In both panels, the indicated values are for 25 animals in the sham-operated control group and 50 animals in the SCAS group at the beginning of the experiment. The Mann–Whitney U-test was used to compare between groups ($p < 0.0001$).

4. Discussion

The procedure herein reported was developed to comprehensively assess the functional state of each mouse before (baseline) and after severe forebrain ischemia. For its development, we considered: (1) that SCAS in mice typically results in severe tissue damage over a large proportion of the brain (Rodriguez et al., 2000), a consistent pattern of mortality, and a wide variety of measurable neurobehavioral alterations, (2) the need for a full clinical characterization of ischemic animals, (3) the recognition that the effectiveness of pharmacological manipulations can be determined only if severity of neurobehavioral deficits can be identified and quantified accurately at two or more points in time to provide a reliable indication of the results of pharmacological interventions and (4) the need for therapeutically relevant measures of neuroprotection.

Our strategy to evaluate functional deficits after brain ischemia has three complementary parts: (a) identification of neurobehavioral alterations produced by SCAS, (b) selection of the most frequent and relevant neurobehavioral alterations produced by SCAS and (c) the use of a general neurological disability status scale.

In this study, with the use of a single procedure, we detected a wide range of neurobehavioral alterations after SCAS. The ischemic syndrome consists of 10 well-defined and consistent alterations: motor incoordination, abnormal body position (lateralized and flattened), hypomobility, decreased body tone and muscular strength, tremor, hunched back, forelimb flexion and ataxic gait. The total neurological scores showed no evidence of recovery from these deficits. On the contrary, data indicate some worsening over time ($p > 0.05$), and the need to extend the period of observation beyond 72 h. It is remarkable that except for ptosis, due to the invasive surgical procedure involving surrounding nerves, which occurred in both sham and SCAS operations, the surgical procedure did not induce significant neurobehavioral changes in sham-operated animals.

Earlier studies in animal models of global ischemia have relied on histological evaluation and placed little emphasis on functional outcome measures. In rats and gerbils, using different tasks, global ischemia is reported to alter spontaneous locomotor activity (Chandler et al., 1985; Gerhardt and Boast, 1988; Wang and Corbett, 1990), responsiveness (Ginsberg and Busto, 1989), body position, righting reflex (Ginsberg and Busto, 1989; Pulsinelli and Brierly, 1979; Bederson et al., 1986), visual paw placement (Schallert et al., 2000), balance on a rotarod (Combs and D'Alecy, 1987; Gionet et al., 1991), learning and memory (Nunn and Hodges, 1994; Block, 1999) and to produce convulsions (Blomqvist et al., 1984; Tsuchiya et al., 2003). In addition, various neurological scoring systems and scales have been employed to measure the severity of the neurological deficit (Nunn and Hodges, 1994; Corbett and Nurse, 1998; Block, 1999; DeVries et al., 2001). However, to date, no single measurement technique has yet proven to be universally acceptable and much of what is done in the laboratory is based on the use of a number of different paradigms for assessing the effects of global ischemia

and possible treatments. A further point is that there are no studies describing the effects of global ischemia in mice, and that only few authors have attempted to describe the complete functional profile of changes after global ischemia in rats (Capdeville et al., 1986). In contrast, a detailed characterization of sensorimotor deficits in mice and rats after focal ischemia has been reported (Hunter et al., 2000; Chen et al., 2001).

The neurological disability status scale described in this article delineates six major progressive steps or grades of overall dysfunction that can be attributed to brain ischemia. This scale is on a 0–10 basis, inasmuch as these represent the sum of the neurological impairments, it permits an equivalent grade for what is hoped to be an equivalent amount of the functional brain damage, regardless of the structures involved. The scale allows for a quantitative measurement of the degree of neurological deficit after brain ischemia and, at the same time, allows for periodic comparisons within or among groups of animals. The relatively high level of neurobehavioral dysfunction and the remarkable difference between sham-operated and SCAS animals, demonstrated in this study, offers the possiblity to test treatment effects and to monitor progress of deficits over time in groups of animals.

Our strategy to produce brain ischemia and to evaluate functional outcome is technically feasible and produces reliable results. However, it has some weaknesses and disadvantages: it is not specific enough to register small changes in individuals; it is laborious, time consuming, requires large sample sizes, consistent experimental conditions, and time to train a person to properly identify normal mice behavior and neurobehavioral alterations. Its major advantage would be its capacity to discriminate relevant neuroprotection (Rodriguez et al., 2003).

In conclusion, this study confirms that SCAS produces a wide range of measurable neurobehavioral deficits in mice. Our evaluation is centered on the motor function, but it also includes evaluation of other brain functions. The developed scale allows for an appropriate quantitative measure of neurobehavioral dysfunction after brain ischemia. We think that this procedure can be essential for neuroprotection studies, allowing to monitor progress of deficits over time in groups of animals.

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DDR

Research Article

Effect of Ascorbic Acid, Dihydrolipoic Acid, t-Butylhydroquinone, and Phenylbutylnitrone on Mortality and Neurological Impairment Induced by Sequential Common Carotid Artery Sectioning in Mice

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Strategy, Management and Health Policy			
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT   The efficacy of four antioxidant agents to reduce neurological deficits and mortality produced by sequential common carotid artery sectioning (SCAS) in mice was evaluated. Ascorbic acid (AA, 500 mg/kg), dihydrolipoic acid (DHLA, 100 mg/kg), tert-butylhydroquinone (t-BHQ, 100 mg/kg) or phenylbutylnitrone (PBN, 100 mg/kg) were injected ip 15 min after the second artery sectioning. Animals were evaluated at 24, 48, and 72 h after antioxidant or saline injection using two procedures: neurological examination and spontaneous motility. Based on neurological examination, a disability status scale was used to determine the degree of functional incapacity after brain ischemia for each animal. The scale comprised 10 progressive steps beyond 0 (normal) extending to status 10 (death due to SCAS). In this scale, the highest scores reflect greater neurological dysfunction. AA and DHLA but neither t-BHQ nor PBN decreased mortality and reduced neurobehavioral deficits caused by SCAS in mice. The observed 24-, 48-, and 72-h survival rates for AA were 86, 71, and 48% and for DHLA were 75, 65, and 65%, representing a significant prolongation of lifespan as compared with vehicle-treated animals (60, 35, 35%). The 24-, 48-, and 72-h neurological scores for AA of 5.2, 6.2, and 7.5 and for DHLA of 4.6, 5.3, and 5.8 as compared with the scores observed for the saline-treated control group (7.6, 8.6, and 8.6) indicate that administration of these antioxidants is beneficial for the neurological output of surviving animals. These antioxidants may have important neuroprotective properties, and indicate that a clinical trial, mainly of DHLA, in stroke patients should be considered. Drug Dev. Res. 63:212–218, 2004.   © 2004 Wiley-Liss, Inc.

Key words: brain ischemia; stroke; mice; antioxidants; neuroprotection

INTRODUCTION

There is considerable evidence that brain ischemia and reperfusion are associated with an excessive formation of nitrogen and oxygen free radicals, including superoxide anion ($O_2 \cdot^-$), hydroxyl ($\cdot OH$), and nitric oxide ($\cdot NO$) [Hall, 1997; Lipton, 1999], which presumably play a central role in neuronal

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injury. The excessive formation of free radicals increases lipid peroxidation in cell membranes, and oxidative damage to DNA and proteins [Chan, 1996]. In support of a major role of reactive oxygen and nitrogen species in ischemia/reperfusion injury is the demonstration that a large number of antioxidants, with different chemical structures and mechanisms of action, are able to reduce lesion volume in models of focal and/or global cerebral ischemia [Hall, 1997]. However, none of these compounds, or other putative neuroprotective agents, has proven to be effective in humans with acute ischemic stroke [Corbett and Nurse, 1998; de Keyser et al., 1999; Green et al., 2003].

There are many reasons for this lack of correlation between preclinical and clinical studies [Wiebers et al., 1990; de Keyser et al., 1999; Dirnagl et al., 1999], among them the understandable limitations of animal models as related to the complexity of human stroke. For instance, major differences exist in the assessment of efficacy in animal experiments measuring histopathological parameters as compared with clinical trials in which the endpoints are mortality and functional status of stroke patients. Therefore, it has been questioned whether reduction in infarct volume in animal experiments is a relevant target for the development of stroke therapy [Corbett and Nurse, 1998].

Because brain injury after stroke in humans is commonly associated with impaired sensorimotor ability, reduced cognitive function, emotional changes, and clinical outcome, it is almost invariably determined through functional parameters, mainly motor ability assessment [Green et al., 2003]. Many laboratories have focused their research in evaluating cognitive and behavioral correlates of histologically determined stroke damage in animal models. It is now considered that assessment of functional status before and after brain ischemia is an alternative strategy to assess potential neuroprotective properties of compounds that are candidates for clinical trials in stroke patients [Rodriguez et al., 2003a].

We reported recently that in mice with previous left common carotid artery sectioning, the addition of right carotid sectioning, 32 days later, elicits a wide range of neurobehavioral alterations, extensive brain damage (cerebral cortex, striatum, and hippocampus), and a high mortality rate [Rodriguez et al., 2000]. We have also described a procedure to identify the neurobehavioral alterations that occur in mice after brain ischemia induced by sequential common carotid artery sectioning (SCAS) in mice, and a strategy to quantify the functional status of ischemic animals and to evaluate neuroprotection [Rodriguez et al., 2003a,b].

In view of the evidence implicating oxidative stress in brain damage by ischemia and reperfusion, and the incomplete information on the neuroprotective properties of antioxidants, in this study we tested the capacity of ascorbic acid (AA), dihydrolipoic acid (DHLA), *tert*-butylhydroquinone (*t*-BHQ) and α-phenyl-*N*-tert-butylnitrone (PBN) to decrease mortality and neurobehavioral deficits induced by SCAS.

MATERIALS AND METHODS

Animals

Studies were carried out in male CFW, aged (40–60 weeks old) mice, weighing 38–57 g at the beginning of the experiments, obtained from our breeding facilities. Animals were housed three to five per cage (same litter) in a temperature-controlled room ($22 \pm 2°C$, relative humidity $55 \pm 3\%$) with an automatically timed cycle of 12-h light/dark (lights on 08:00–20:00). Food (Purina Chow, St. Louis, MO) and water were available ad libitum. Mice were allowed to acclimate to the environmental conditions for at least 1 week prior to experimentation. Twelve hours before experiments, food was withheld and free access to water was maintained. Surgery and experiments were performed between 09:00 and 14:00 h. All experiments were carried out in accordance with the Declaration of Helsinki, and adhered to the National Health Ministry of Mexico guidelines for the use of laboratory animals.

Procedures

Brain ischemia was produced as described previously [Rodriguez et al., 2000]. Briefly, light anesthesia was induced with ether delivered by facemask and the left common carotid artery was exposed through a midline neck incision and dissected free from surrounding nerves and fascia. The artery was sectioned between ligatures and the incision was closed with surgical thread. After surgery animals, were kept in a recovery room for about 1 h under heat lamps to maintain body temperature. Once animals regained complete consciousness, they were moved to the environmentally controlled room. Animal weight was monitored daily. Thirty-two days later, mice were clinically evaluated using the procedure described below. Mice showing any degree of neurological deficit were excluded from the following experiments. Groups of 22–23 animals were randomly assigned into one of the different experimental groups prior to the induction of anesthesia and sectioning of the right common carotid artery, and numbered (dye marking). Four groups of animals were studied: mice not submitted to anesthesia and surgery, sham-operated, SCAS

with vehicle treatment, and SCAS with antioxidant treatment.

Mortality

Deaths were scored continuously over the first 6 h after the second surgery and then every 6 h during the first 24 h. Thereafter, deaths were scored every 12 h up to the end of the experiment (72 h). The number of deaths and the time of death were used to calculate survival curves.

Neurobehavioral Testing

We used two procedures to quantify the neurobehavioral alterations produced by SCAS: neurological examination and spontaneous motility. Testing was performed immediately before (baseline) and 24, 48, and 72 h after the right carotid artery occlusion. At any given day, the sequence of testing was spontaneous motility and neurological evaluation. All experiments were carried out at room temperature. In each assay, animals were run in a randomized order.

Neurological Examination and the Neurological Disability Status Scale (NDSS)

The procedure used in this study for the neurological assessment of mice has been previously described [Rodriguez et al., 2003a,b], and is essentially an adaptation of the one validated by Irwin [1968]. Briefly, the neurological examination consisted of an initial phase of undisturbed observation and a later manipulative phase during which each animal is submitted to a sequence of maneuvers, starting with the least disturbing. Observations and manipulations were specifically directed to identify the presence or absence of the more characteristic and consistent elements of the ischemic syndrome induced by SCAS: hypomotility, passivity, abnormal posture, hunched back, ataxic gait, tremor/twitches/convulsions, respiratory distress, circling, decreased body tone, muscle weakness, front limb paralysis, and motor incoordination. Except for two parameters, the occurrence of any of these alterations was scored with a 0 or 1 (0=not present, 1=present). Spontaneous motility and motor coordination were graded in terms of their affected level on a scale of 0–3 (0=normal, 1=slightly decreased, 2=markedly decreased, 3=total incapacity to move). The same observer, without prior knowledge of the respective treatments, conducted all neurological assessments.

The details of the scale used to determine the degree of functional incapacity after brain ischemia were published elsewhere [Rodriguez et al., 2003a]. Briefly, the NDSS comprises 10 progressive steps beyond 0 (normal), extending to status 10 (death due to SCAS). Therefore, the higher the number, the greater is the neurological dysfunction. Accordingly, the six major steps indicate 0 for normal (no neurological dysfunction) and 2 for slight decrease in mobility and the presence of passivity. Category 4 represents moderate neurological dysfunction and includes findings such as flattened/lateralized posture, hunchback, ataxic gait, tremors, decreased body tone, muscle weakness, and slight motor incoordination. Category 6 represents animals more handicapped but still able to walk, with findings such as marked hypomotility, circling, jerks or convulsions, forelimb flexion, and marked motor incoordination. Category 8 indicates total incapacity to move and respiratory distress. Status 10 corresponds to death due to SCAS.

Spontaneous Motility

The spontaneous motility of animals in a novel environment (mice were naive to the locomotor chamber) was measured with an Opto-Varimex animal activity meter (Columbus Instruments, Columbus, OH), which monitored only the horizontal (locomotion) movements of animals. The individual compartments ($43.2 \times 44.4 \times 20$ cm, clear plastic sidewalls and cover) were equipped with 15 infrared light beams on each axis (beam spacing, 2.65 cm; beam diameter, 0.32 cm). External timer-controlled counters recorded the number of counts representing all light beam interruptions of any of the sensors. Mice were individually tested in a dimly lit, quiet room. They were removed from their home cages and placed in the middle of an activity monitor, and the data-collecting system was immediately activated. Testing was done immediately before (baseline) and at 24, 48, and 72 h after the second surgery. Motility scores are the number of beam interruptions per 1-min test session.

Pharmacology Testing

Mice were injected ip with AA (500 mg/kg), DHLA (100 mg/kg), t-BHQ (100 mg/kg), or PBN (100 mg/kg) 15 min after the second arterial section; control groups received saline. Deaths were scored at the indicated times, and survivors were evaluated as described above. Doses of antioxidants were those reported to limit infarct size after experimental cerebral ischemia [Prehn et al., 1992; Stamford et al., 1999; Yang et al., 2000].

Compounds

All compounds were obtained from Sigma, St. Louis, MO, and prepared fresh before use in 0.9% saline; doses are expressed in terms of the salts. Compounds were given ip in a volume of 0.1 mL/10 g body weight.

Data Analysis

The survival curves were calculated by the Kaplan-Meier method and compared using the log-rank test-tailed. Neurological scores for the different treatment groups at the various times were compared to the saline-treated group by the Kruskal-Wallis analysis of variance (nonparametric ANOVA). After a significant ANOVA, Dunn's test was used to compare all treated groups with the saline control. Locomotion and body weight data were analyzed by one-way ANOVA. After significant ANOVA, Dunnett's test was used to compare treatment groups with the saline control. In all cases, a probability value less than 0.05 indicated statistical significance. Analyses were done using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Effects on Mortality

No deaths were observed in control animals (no anesthesia, no surgery), and only one death was observed in sham-operated mice (1/14). Some deaths were observed immediately after right carotid sectioning, and mortality among groups ranged from 4.8 to 26% (average, 12.4%) within the first 15 min after second surgery, before antioxidant or saline administration. These deaths were not taken into account for the analyses. Figure 1 shows the Kaplan-Meier survival curves for sham-operated, saline-treated, and drug-treated animals. Sham-operated animals showed a 93% survival rate throughout the study period of 72 h. The overall 24-, 48-, and 72-h survival rates of saline-treated animals were 60%, 35%, and 35%.

The administration of AA and DHLA clearly reduced the number of deaths of animals submitted to SCAS (Fig. 1). The log-rank test indicated that the survival curve is significantly different for AA-treated animals at 24 and 48 h. In the case of DHLA, the increase in survival did not reach statistical significance ($P=0.05$). t-BHQ and PBN did not protect animals against death.

Effects on Neurobehavioral Alterations

None of the sham-operated animals showed detectable neurobehavioral abnormalities, whereas in the surviving saline-treated animals, a consistent pattern of neurological alterations was observed. Figure 2 shows the detailed neurological scoring of antioxidant-treated animals in comparison with sham-operated and saline-treated groups. Treatment with AA and DHLA consistently decreased total neurological scores. At 24 and 48 h, the scores obtained with AA and
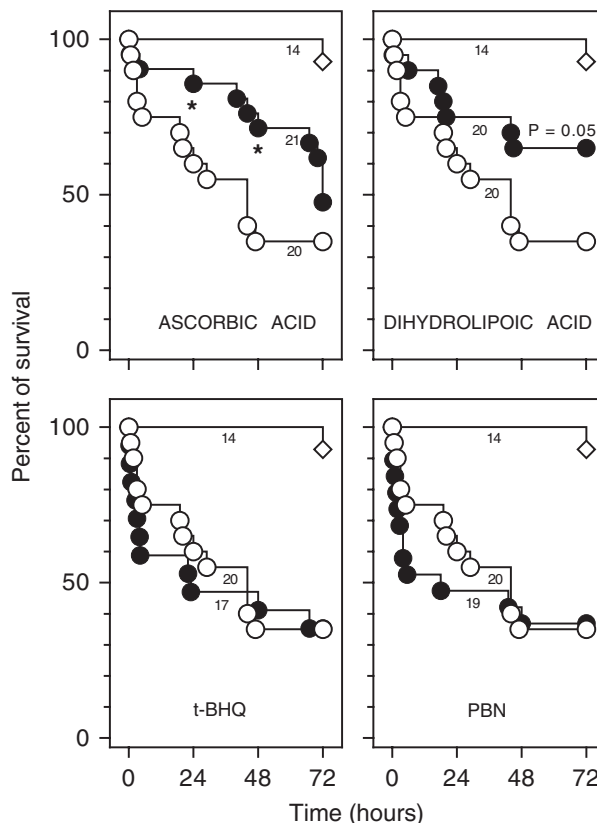

Fig. 1. Effect of antioxidants on the mortality induced by sequential carotid artery sectioning in mice. Each panel shows survival curves for sham-operated (◇), saline-treated (○), and drug-treated (●) animals. Ascorbic acid (500 mg/kg), dihydrolipoic acid (100 mg/kg), t-butylhydroquinone (100 mg/kg), phenylbutylnitrone (100 mg/kg), or saline were given ip 15 min after the second surgery. Deaths were scored continuously over a period of 6 h and thereafter every 12 h up to 3 days. Data are derived from a variable number of animals (n=14–21) at the beginning of the experiment, as indicated for each curve. The abscissae represent time after antioxidant administration; the ordinates percent survival. The survival curves were calculated by the Kaplan-Meier method and compared using the log-rank test. Asterisks denote differences from the saline-treated group ($P<0.05$).

DHLA were significantly lower that those of the saline-treated animals. For DHLA, the difference at 72 h was only marginally significant. Neither t-BHQ nor PBN administration decreased neurological scores.

Motility gradually decreased in sham-operated mice with repeated testing, but reductions were significantly different at 72 h when compared with the control baseline (Fig. 3). Except at one point (48 h), motility of saline-treated animals was significantly lower than that observed in sham-operated mice. Motility of mice treated with DHLA was consistently higher than that recorded with the saline-treated group, but significant difference was validated only at 48 h. There were no differences in motility among
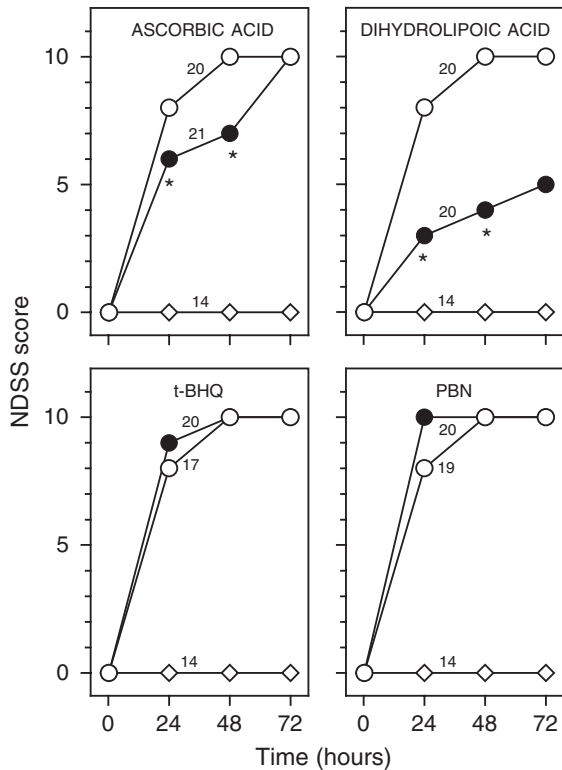
Fig. 2. Effect of antioxidants on the neurological dysfunction induced by sequential carotid artery sectioning in mice. Each panel shows the median neurological scores for sham-operated (◇), saline-treated (○), and antioxidant-treated (●) animals. Ascorbic acid (500 mg/kg), dihydrolipoic acid (100 mg/kg), *t*-butylhydroquinone (100 mg/kg), or phenylbutylnitrone (100 mg/kg) or saline were given ip 15 min after the second surgery. The degree of neurological impairment was rated on six major progressive steps: 0 represents no neurological dysfunction; 2 indicates minimal disability; 4 represents moderate dysfunction; category 6 represents more handicapped animals; category 8 refers severe disability; and status 10 indicates death. The abscissae represent time after antioxidant administration; the ordinates represent the grade of neurological dysfunction. Symbols correspond to medians of neurological scores for 14–21 animals, as indicated for each curve. Asterisks denote significant reduction ($P < 0.05$) in Neurological Disability Status Scale (NDSS) score as compared with the saline-treated animals (Kruskal-Wallis followed by Dunn's test).

animals receiving AA, *t*-BHQ, or PBN and those injected with saline.

All operated animals lost body weight. Sham-operated mice lost almost 1.7 g, on average, as compared to their preoperative weight. In contrast, vehicle-treated mice subjected to SCAS lost a significant proportion of their body weight. The mean difference between presurgical body weight and weights at 24, 48, and 72 h after the second surgery was 5.4 ± 1.1, 6.5 ± 1.3, and 8.5 ± 1.6 g, respectively. None of the test compounds influenced the loss of body weight produced by SCAS.
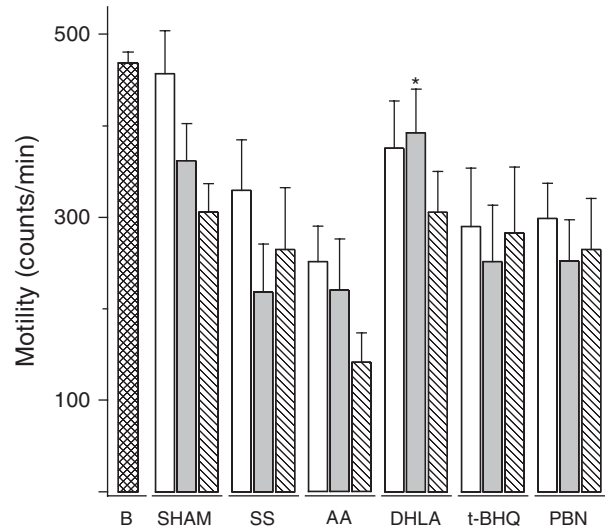

Fig. 3. Effect of antioxidants on the hypomotility induced by sequential carotid artery sectioning in mice. The criss-cross bar represents the mean of baseline counts. Each group of three bars represents motility at 24 (empty), 48 (speckled), and 72 (diagonal) h after the second carotid artery sectioning. Ascorbic acid (AA) (500 mg/kg), dihydrolipoic acid (DHLA) (100 mg/kg), *t*-butylhydroquinone (t-BHQ) (100 mg/kg), or phenylbutylnitrone (PBN) (100 mg/kg) or saline were given ip 15 min after the acute ischemic event. Spontaneous motility was measured for a 1-min period and each bar represents the mean of a variable number of animals (n=14–21) at the beginning of the experiment. Vertical lines indicate standard errors. *$P < 0.05$, compared to the corresponding bar of saline-treated animals (analysis of variance followed by Dunnett's test).

DISCUSSION

In animal models of global and focal ischemia, neuroprotection is commonly assessed by histological measures (e.g., cell loss or infarct volumes). The limitations of a purely histological approach in assessing neuroprotection are clearly illustrated by the fact that a large number and variety of compounds have proven highly effective in reducing cell loss and infarct volume in experimental animals [Read et al., 1999; Green et al., 2003], but all have failed in human trials of acute ischemic stroke [Corbett and Nurse, 1998; de Keyser et al., 1999; Green et al., 2003].

As previously mentioned, many studies have demonstrated the efficacy of different antioxidants, including AA [Stamford et al., 1999; Henry and Chandy, 1998], DHLA [Prehn et al., 1992; Panigrahi et al., 1996; Cao and Phillis, 1995; Wolz and Krieglstein, 1996] and PBN [Phillis and Clough-Helfman, 1990; Cao and Phillis, 1994; Yang et al., 2000; Li et al., 2001], to reduce ischemic brain damage in models of global/forebrain ischemia in gerbils, as well as in models of both permanent and transient of focal ischemia in rats and mice. However, few studies have examined the effect of these antioxidants on mortality

[Floyd, 1990; Panigrahi et al., 1996; Li et al., 2001] and/or functional outcome after ischemia [Cao and Phillis, 1995; Woltz and Krieglstein, 1996; Yang et al., 2000], and these have used only one measure of functional deficit (e.g., locomotor activity) to evaluate antioxidant neuroprotection.

In this study, we found that AA and DHLA, but not t-BHQ nor PBN, decreased mortality and neuro-behavioral deficits caused by SCAS in mice. DHLA also reversed hypolocomotion. These effects represent a remarkable property of AA and DHLA because other putative neuroprotective agents, such as deferoxamine, dizocilpine, and nimodipine, have failed to reduce mortality and/or consistently diminish neurological deficits that occur after SCAS [Rodriguez et al., 2003a]. The findings with AA and DHLA are compatible with previous data showing that both antioxidants ameliorate brain ischemic damage. DHLA has also been reported to markedly reduce mortality induced by bilateral carotid artery occlusion and hypotension in rats [Panigrahi et al., 1996].

The pharmacological mechanisms responsible for the neuroprotective effects of AA and DHLA against mortality and neurological deficits produced by SCAS have not been clarified in this study, although they may be attributed to their antioxidant activity, as has been the case for their ability to limit infarct size in ischemic animals. AA is a potent scavenger that quenches free radicals by donating two electrons and then becoming oxidized itself to dehydroascorbic acid [Rice, 2000]; it also participates in the recycling of other antioxidants such as vitamin E, which stops peroxidation of cell membranes. It should be noted that AA does not cross the normal blood–brain barrier; thus, the neuroprotection evident in this study may be explained by entry into the brain via a compromised blood–brain barrier [Preston et al., 1993]. DHLA is a thiol antioxidant that crosses the blood–brain barrier. Four antioxidant properties have been demonstrated for this drug: metal chelating capacity [Scott et al., 1994]; reactive oxygen species (ROS) scavenging [Kagan et al., 1992]; regeneration of endogenous antioxidants, including glutathione and vitamins E and C [Packer et al., 1995]; and repair of oxidative damage [Biewenga et al., 1997]. In addition, it modulates transcription factor activities, especially that of NF-κB [Packer et al., 1997].

The negative results with PBN contrast with previous studies indicating that this antioxidant reduces infarct volume in rats and gerbils subjected to both focal and global ischemia [Cao and Phillis, 1994]. PBN is also a potent free radical scavenger. It reacts with oxygen- and carbon-based free radicals to form stable adducts [Yang et al., 2000; Kotake, 1999]. There are several possible explanations for the discrepancy between our findings with PBN and those reported by other authors. First, and most important, are methodological issues concerning the experimental model and the criteria used to measure the effects of brain ischemia and the degree of neuroprotection. SCAS is essentially a model of global ischemia with one important difference. In most models of forebrain ischemia, occlusion of the carotid arteries is only transient, and both the onset of ischemia and its subsequent reversal are abrupt [Ginsberg and Busto, 1989]. In our model, sectioning the right common carotid artery in animals subjected to significant reduction of blood supply by previous left common carotid artery sectioning produces acute forebrain ischemia. We postulated that this model might be relevant to stroke in humans, since it implies provoking an acute ischemic insult in animals already submitted to chronic hypoperfusion, as occurs in humans, and uses neurological and survival endpoints, instead of infarct volume, to determine the effects of brain ischemia and the degree of neuroprotection. Another important consideration is that previous studies with PBN were conducted in healthy, young to middle-aged animals. Differences in susceptibility to brain ischemia between young and aged animals have been clearly established [Yager et al., 1996; Fuentes-Vargas et al., 2002]. It is quite possible that treatments found effective in young animals may not necessarily be so in aged animals.

The failure of t-BHQ and PBN in our experimental model of brain ischemia pinpoints the possibility that the efficacy of AA and DHLA is related to their capacity to interrupt at different points of the oxidative stress mechanisms in brain ischemia. Clearly, a variety of additional processes may exist to explain the neuroprotective properties of these antioxidants [Clemens, 2000; McCarty, 2001], although the present results give no clue as to the nature of such mechanisms.

Finally, this work confirms that SCAS leads to a consistent pattern of measurable functional impairments in mice, and that our procedure to determine the severity of functional deficits is a valuable tool in the search for possible neuroprotective effects of pharmacological interventions.

In summary, administration of AA and DHLA increases survival and decreases neurological alterations in mice subjected to SCAS. These findings strongly support the idea that these drugs, mainly DHLA, could have potentially useful neuroprotective activity in stroke patients. Our strategy to evaluate neuroprotective efficacy is sufficiently sensitive to detect differences among members of the same group of drugs. These results confirm the validity and usefulness of our experimental stroke model.

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Research Article

Outstanding Neuroprotective Efficacy of Dexrazoxane in Mice Subjected to Sequential Common Carotid Artery Sectioning

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT   We evaluated the efficacy of the iron chelator, dexrazoxane, to reduce the neurological alterations and mortality produced by sequential common carotid artery sectioning (SCAS) in mice. Deferoxamine (32 and 128 mg/kg), dexrazoxane (32 and 128 mg/kg), dizolcipine (0.8 and 3.2 mg/kg), or nimodipine (0.4 and 1.6 mg/kg) were injected ip 15 min after the second artery sectioning. Animals were evaluated at 24, 48, and 72 h after compound or vehicle injection using three procedures: neurological examination, spontaneous locomotor activity, and motor coordination. Based on neurological evaluation, we designed and used a disability status scale (NDSS) to determine the degree of functional incapacity after brain ischemia for each animal. The scale comprises 10 progressive grades or steps beyond 0 (normal), extending to status 10 (death due to SCAS). In this scale, the higher the number, the greater is the neurological dysfunction. For the vehicle-treated animals, the survival rate ranged from 41 to 30% and the neurological scores ranged from 6.6 to 7.8 between 24 and 72 h after the second surgery. The survival rate was significantly increased with low and high doses of dexrazoxane (ranges, 57–52% and 66–60%, respectively). Total neurological scores with 32 and 128 mg/kg of dexrazoxane (ranges, 4.5–5.4 and 3.3–4.0, respectively) were significantly lower ($P < 0.05$) than its corresponding solvent. Dexrazoxane administration also avoided hypolocomotion and motor incoordination produced by SCAS. None of three known putative neuroprotective agents tested in this study reduced the mortality and/or consistently diminished neurological deficits. We conclude that dexrazoxane has very important neuroprotective properties and that our scale is useful to quantify the degree of neurological impairment after brain ischemia. Drug Dev. Res. 60:294–302, 2003.   © 2003. Wiley Liss, Inc.

Key words: brain ischemia; mice; stroke; dizolcipine; dexrazoxane; deferoxamine; nimodipine

INTRODUCTION

Animal experiments have shown the existence of a large number of compounds with different mechanisms of action, from calcium channel and glutamate receptor antagonists to NO-pathway inhibitors and free-radical scavengers [Read et al., 1999] that reduce tissue damage after brain ischemia. However, despite having been proven effective in traditional models of

cerebral ischemia, none of these compounds has demonstrated unequivocal efficacy when administered after stroke in humans [De Keyser et al., 1999]. Therefore, the value of such models of brain ischemia in the discovery and development of neuroprotective agents has been severely questioned [Wiebers et al., 1990; Hunter et al., 1995; De Keyser et al., 1999], and substantial efforts are being made to develop models and strategies to detect clinically effective compounds.

We reported recently that in mice with previous left common carotid artery sectioning, the addition of right carotid sectioning, 32 days later, elicits profound neurological alterations, brain damage, and a high mortality rate [Rodriguez et al., 2000a]. This new model of experimental ischemic stroke is essentially a model of global ischemia with one important difference. In most models of forebrain ischemia, occlusion of the carotid arteries is only transient, and both the onset of ischemia and its subsequent reversal are abrupt [Ginberg and Busto, 1989]. In our model, acute forebrain ischemia is produced by sectioning the right common carotid artery (CCA) in animals subjected to a significant reduction of blood supply by previous left CCA sectioning. We postulated that this model of global ischemia might be relevant to stroke in humans. It incorporates the idea of provoking an acute ischemic insult in animals subjected to chronic hypoperfusion (comorbid condition) and uses neurological and survival endpoints to determine the effects of brain ischemia and the degree of neuroprotection.

Using the sequential common carotid artery sectioning (SCAS), we found that dexrazoxane, a powerful iron chelator, substantially reduces the cumulated mortality rate in mice subjected to severe forebrain ischemia [Rodriguez et al., 2000b]. We also observed that dexrazoxane reduces the number of animals with severe neurological dysfunction. In view of the large individual variations, and the wide range of neurobehavioral changes induced by SCAS, we did not attempt to categorize and quantify these alterations; only their incidence was recorded. We considered it pertinent to develop strategies, standards, and criteria to identify functional deficits after SCAS and to design an index of functional disability that would allow us to quantify the consequences of brain ischemia and guide the selection of promising candidates to take into clinical trials.

The present experiments were aimed at assessing the neuroprotective activity of dexrazoxane and comparing it with three well-known putative neuroprotective agents that act through different mechanisms of action. In addition, we determined whether the procedure described in this report could effectively rate the neurological status of ischemic animals and discriminate protection of compound-treated animals. Since there are age-related differences in response to brain ischemia provoked by SCAS [Fuentes-Vargas et al., 2002], the present study was conducted in aged animals.

MATERIALS AND METHODS

Animals

Studies were performed in male CFW, aged (40–60 weeks old) male mice, weighing 38–57 g at the beginning of the experiments, obtained from our breeding facilities. Animals were housed 3–5 per cage (same litter) in a temperature-controlled room ($22\pm2°C$, relative humidity $55\pm3\%$) with an automatically timed cycle of 12/h light/dark (lights on 08:00–20:00). Food (Purina Chow, St. Louis, MO) and water were available ad libitum. Mice were allowed to acclimate to these environmental conditions for at least one week prior to experimentation. Twelve hours before experiments, food was withheld but free access to water was maintained. Surgery and experiments were performed between 09:00–15:00 h during the light period. This study was carried out with permission of the local animal care and use committee under the provisions of the Declaration of Helsinki.

Procedure

Brain ischemia was produced as described previously [Rodriguez et al., 2000a]. Briefly, light anesthesia was induced with ether delivered through a facemask, and the left common carotid artery was exposed through a midline neck incision and dissected free from surrounding nerves and fascia. The artery was sectioned between ligatures and the incision was closed with surgical thread. After surgery, animals were kept in a recovery room for about 1 h under heat lamps to maintain body temperature. Once animals regained complete consciousness, they were moved to the environmentally controlled room. The animals' weight was monitored daily. Thirty-two days later, mice were clinically evaluated using the procedure described below. Mice showing neurological deficit of any degree were excluded from the following experiments. Groups of 18–22 animals were randomly assigned to the different experimental groups prior to the induction of anesthesia and sectioning of the right common carotid artery and numbered (dye marking). Mice not subjected to anesthesia and surgery, and sham-operated mice undergoing anesthesia and full surgical procedures, except for artery ligation and section, served as controls.

Mortality

Mortality was scored continuously over the first 6 h after the second surgery and then every 6 h during the first 24 h. Thereafter, every 12 h up to the end of the experiment (72 h).

Neurobehavioral Testing

We used three procedures to quantify the neurobehavioral alterations produced by the sequential carotid artery sectioning (SCAS): neurological examination, spontaneous locomotor activity, and motor coordination. Neurobehavioral evaluations were performed immediately before and 24, 48, and 72 h after the right carotid artery sectioning. At the corresponding day, the sequence of testing was spontaneous locomotor activity, neurological evaluation, and motor coordination. All evaluations were performed at room temperature. In each test, animals were run in a randomized order, and control mice were run concurrently with animals treated with each dose of the compound being tested.

Neurological examination and the neurological disability status scale (NDSS)

The procedure used was essentially based on the one validated by Irwin [1968] and involves an initial phase of undisturbed observation followed by a manipulative phase during which each animal is subjected to a sequence of manipulations, starting with the least provoking stimuli. Based on our previous studies [Rodriguez et al., 2000a], indicating that SCAS produces a wide variety and different degrees of neurological alterations, and due to the inconsistencies found in some of them, observations and manipulations were specifically directed to identify the presence or absence of the most frequent and relevant neurological alterations induced by SCAS. As indicated in Table 1, a total of 14 different neurological alterations were recorded for each animal. Except for two parameters, the occurrence of any of these alterations was scored with 0 or 1 (0 = not present, 1 = present). Spontaneous mobility and motor coordination were graded in terms of their level of affectation on a scale 0–3 (0 = normal, 1 = slightly decreased, 2 = markedly decreased, 3 = total incapacity to move and coordinate). With this strategy, the neurological examination of each animal can be performed in 3 to 4 min. The observer that conducted the neurological assessment had no knowledge of the procedure that the animal had undergone.

Based on the neurological examination, we designed and used an overall disability status scale (NDSS) to depict the presence, severity, and progression of the functional impairment after brain ischemia.

TABLE 1. Most Frequent and Relevant Neurological Alterations Observed After SCAS in Mice and Their Distribution According to the Degree of Neurological Disability

Neurological alterations[a]	Neurological disability status scale (NDSS)	
Hypomobility	0=	Normal
Flattened posture	2=	Hypomobility (slight)
Lateralized posture		Passivity
Hunched back	4=	Hypomobility (moderate)
Piloerection		Flattened posture
Ataxic gait		Lateralized posture
Tremor/twitches/ convulsions		Hunched back
Respiratory distress		Ataxic gait
Circling		Piloerection
Passivity		Tremors
Decreased body tone		Decreased body tone
Muscle weakness		Muscle weakness
Front limb flexion		Motor incoordination (slight)
Motor incoordination (tight cord, inclined plane)	6=	Hypomobility (marked)
		Circling
		Twitches/convulsions
		Front limb flexion
		Motor incoordination (moderate)
	8=	Hypomobility (severe)
		Motor incoordination (severe)
		Respiratory distress
	10=	Death

[a]Arranged in the order in which they were evaluated.

This scale has 10 progressive grades or steps beyond 0 (normal), extending to status 10 (death due to SCAS); therefore, the higher the number, the greater the neurological dysfunction (Table 1). Accordingly, the six major steps indicate: zero for normal (no neurological dysfunction) and 2 represents a slight decrease in mobility and the presence of passivity. Category 4 represents moderate neurological dysfunction and includes findings such as moderate hypomobility, flattened posture, hunched back, lateralized posture, ataxic gait, tremors, decreased body tone, muscle weakness, and slight motor incoordination. Category 6 corresponds to more handicapped animals but still able to walk, with marked hypomobility, circling, jerks or convulsions, front limb flexion, and moderate motor incoordination. Category 8 corresponds to respiratory distress and total incapacity to move/coordinate. Status 10 corresponds to death due to SCAS. In all cases, where criteria for the precise grade were not met, the nearest appropriate number was used (1, 3, 5, 7, 9). This scale allowed for periodic comparisons within or among groups of animals.

Spontaneous locomotor activity.

The animals' spontaneous locomotor activity (motility) in a novel environment (mice were naive to

the locomotor chamber) was measured with the Opto-Varimex animal activity meter (Columbus Instruments, Columbus OH), which monitored only the horizontal (locomotion) movements of the animals. The individual compartments ($43.2 \times 44.4 \times 20$ cm, clear plastic side-walls and cover) were equipped with 15 light beams on each axis (beam spacing, 2.65 cm; beam diameter, 0.32 cm). External time-controlled timers recorded the number of counts representing all light beam inter-ruptions of any of the sensors. Mice were individually tested in a dimly lit, quiet room. They were removed from their home cages and placed in the middle of an activity monitor and the data-collecting system was simultaneously activated. Testing was done immedi-ately before the second surgery (baseline) and at 24, 48, and 72 h after the second surgery. Spontaneous motor activity was registered for 2-min sessions and expressed as percentage of the activity of each animal during the preoperative session (baseline).

Motor coordination

Possible motor impairment was assessed with the accelerating rotarod test. Before the second surgery, animals were trained to remain for at least 120 sec on a rotarod (Ugo Basile, Varese, Italy) set at a constant speed of 8 rpm. On the day of and before the second surgery, the latency of mice to fall from an accelerating (3–30 rpm over a period of 120 sec) rotarod was recorded and used as the baseline score for each animal; 24, 48, and 72 h after the second surgery, each mouse was tested again on the accelerated rotarod. Results for each mouse at each time point were expressed as percentage of their presurgery baseline time.

Pharmacology Testing

Mice were injected i.p. with deferoxamine (32 and 128 mg/kg), dexrazoxane (32 and 128 mg/kg), dizolcipine (0.8 and 3.2 mg/kg), or nimodipine (0.4 and 1.6 mg/kg) 15 min after the second arterial section; control groups received the corresponding vehicle or solvent. Mortality was scored at the indicated times and survivors were evaluated as described above.

Compounds

Deferoxamine, dizolcipine, and nimodipine were obtained from Sigma (St. Louis, MO). Dexrazoxane was a gift from Pharmacia and Upjohn, Mexico. Compounds were prepared fresh before being used and suspended in 0.2% methylcellulose or in its solvent (dexrazoxane); doses are expressed in terms of their salts. Injections were applied i.p. in a volume of 0.1 ml/10 g body weight.

Data Analysis

The survival curves were calculated with the Kaplan-Meier method and compared using the log-rank test. The Mantel-Haenszel test was used to compare treatment groups with the corresponding vehicle. Differences between total neurological scores for different doses of the compounds at the different periods of time, as compared to their respective data for the vehicle, were calculated by the Kruskal-Wallis analysis of variance (non-parametric ANOVA). Follow-ing significant ANOVA, Dunn's test was used to compare treatment groups with vehicle control. The parametric behavioral data (spontaneous locomotor activity, rotarod performance) and body weight were initially analyzed through a one-way variance analysis (ANOVA). Following significant ANOVA, Tukey's test was used to compare treatment groups with vehicle control. Analysis was done using GraphPad Prism version 3.02 for Windows (GrapPad Software, San Diego, CA).

RESULTS

Mortality

No deaths were observed in control animals (no anesthesia no surgery) and, except for two cases (2/64), no deaths were observed in sham-operated mice. Some deaths were observed immediately after the right carotid sectioning, and mortality ranged from 16 to 36% (average, 26%) within the first 15 min after surgery, before compound or vehicle administration. These deaths were not taken into account for analysis. Figure 1 shows the corresponding Kaplan-Meier survival curves for sham-operated and vehicle-treated animals in each group of experiments. Sham-operated animals demonstrated 96% survival (range, 93–100%) throughout the study period of 72 h. The overall 24, 48, and 72 h survival rates of vehicle-treated animals were 41% (range, 36 to 50%), 35% (range, 29 to 44%), and 30% (21–38%), respectively. No statistically significant difference in survival rates was found among the three different vehicle-treated groups.

Dexrazoxane (32 and 128 mg/kg) significantly reduced the number of deaths of animals subjected to SCAS, as compared with its respective vehicle-treated group. In animals treated with 32 mg/kg the 24, 48, and 72 h survival rates were 57, 52, and 52%, respectively. For 128 mg/kg, the survival rates were 66, 66, and 60%, at 24, 48, and 72 h, respectively. These data clearly contrast with the 38, 28, and 21% survival rates observed, for the same points of time, with its vehicle-treated group. The results of the log-rank test indicated that the median survival times of both dexrazoxane-treated groups are significantly different
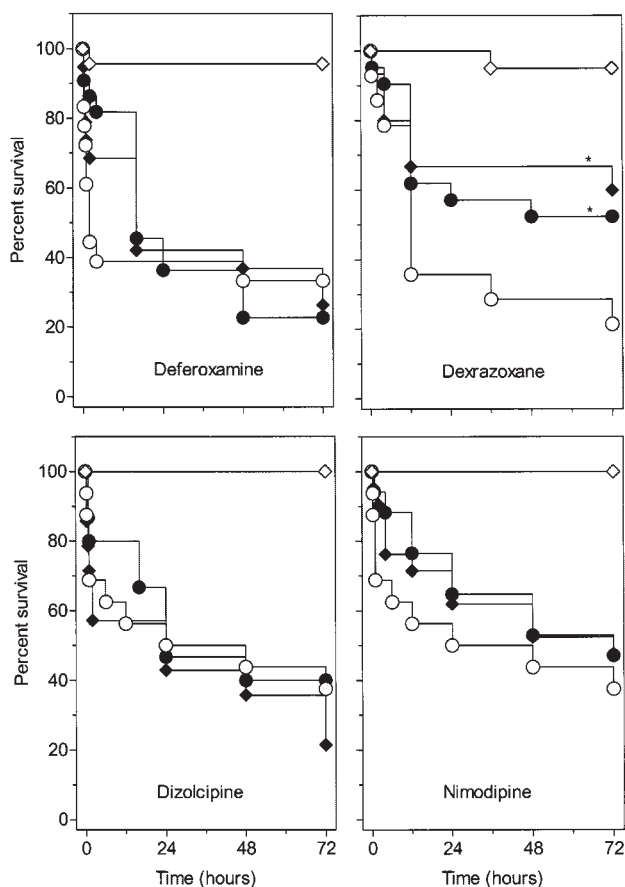
Fig. 1. Effect of dexrazoxane and reference neuroprotective agents on the mortality induced by sequential carotid artery sectioning in mice. Each panel shows survival curves for sham-operated (open diamonds), vehicle-treated (open circles) and compound-treated animals. In each panel, closed circles and diamonds indicate low and high doses, respectively, of deferoxamine (32 and 128 mg) dexrazoxane (32 and 128 mg/kg), dizolcipine (0.8 and 3.2 mg/kg), or nimodipine (0.4 and 1.6 mg/kg). Compounds or the corresponding vehicle were given i.p. 15 min after the second surgery. Deaths were scored continuously over a period of 24 h and thereafter every 12 h up to 3 days. Data are derived from a variable number of animals (n = 14–22) at the beginning of the experiment. The abscissae represent time after compound administration; the ordinates, percent survival. The survival curves were calculated by the Kaplan-Meier method and compared using the log rank test. The Mantel-Haenszel test was used to compare treatment groups with vehicle control. *Different from its vehicle-treated group ($P < 0.05$).

Fig. 2. Effect of dexrazoxane and reference neuroprotective agents on the neurological dysfunction induced by sequential carotid artery sectioning in mice. Each panel shows the mean neurological scores for sham-operated (open diamonds), vehicle-treated (open circles) and compound-treated animals. In each panel, solid circles and diamonds indicate low and high doses, respectively, of deferoxamine (32 and 128 mg/kg) dexrazoxane (32 and 128 mg/kg), dizolcipine (0.8 and 3.2 mg/kg), or nimodipine (0.4 and 1.6 mg/kg). Compounds or the corresponding vehicle were given i.p. 15 min after the second surgery. The degree of neurological impairment was rated on 6 major progressive steps: 0 represents no neurological dysfunction; 2 indicates minimal disability; category 4 represents moderate dysfunction; category 6 represents more handicapped animals; category 8 refers severe disability; and status 10 indicates death due to SCAS. The abscissae represent time after compound administration, the ordinates the grade of neurological dysfunction. Values are the means ± S.E.M. of the neurological scores for 14–22 animals. *Significant reduction ($P < 0.05$) in NDSS score as compared with the respective vehicle-treated group (Kruskal-Wallis followed by Dunn's test).

from the vehicle-treated group. Neither of the three reference compounds (deferoxamine, dizolcipine, or nimodipine) protected animals against death.

Neurobehavioral Alterations

None of the sham-operated animals showed significant neurological abnormalities, whereas in the surviving vehicle-treated animals, a consistent pattern of neurological alterations was observed (Table 1). Figure 2 shows the detailed neurological scoring with
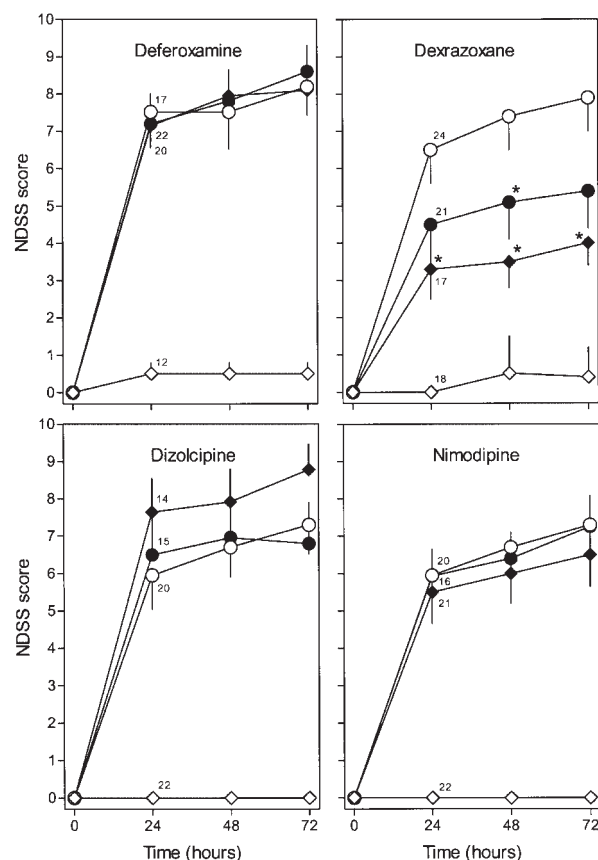
different doses of the tested compounds in comparison with their respective vehicle-treated group. The mean NDSS scores for vehicle-treated animals were 6.6 (range, 5.9–7.5) at 24 h, 7.2 (range, 6.8–7.5) at 48 h, and 7.8 (range, 7.3–8.2) at 72 h. This tendency to increase was not statistically significant. With dexrazoxane, there was a consistent decrease in total neurological scores. At all times, scores obtained with 128 mg/kg were

significantly lower that those of the solvent-treated mice. The lower dose of dexrazoxane also decreased neurological scores but the difference was only significant at 48 h. The two doses of deferoxamine and nimodipine showed no significant differences when compared with their vehicle groups. The higher dose of dizolcipine (3.2 mg/kg) consistently increased neurological scores as compared with its vehicle-treated group, although differences were not statistically significant.

In sham-operated animals, the overall locomotion gradually decreased with repeated testing (78, 65, and

47% of the baseline); these reductions were significantly different when compared with their respective control baselines. Except at one point (72 h), locomotion of vehicle-treated animals was significantly lower than that observed in sham-operated mice (Fig. 3). Locomotion of animals treated with dexrazoxane was consistently higher than that recorded with its solvent-treated group. A significant difference in locomotion between animals receiving dexrazoxane (128 mg/kg) and the solvent-treated group was found at 24 and 48 h. In fact, locomotion of dexrazoxane-treated animals was not statistically different from that of sham-operated
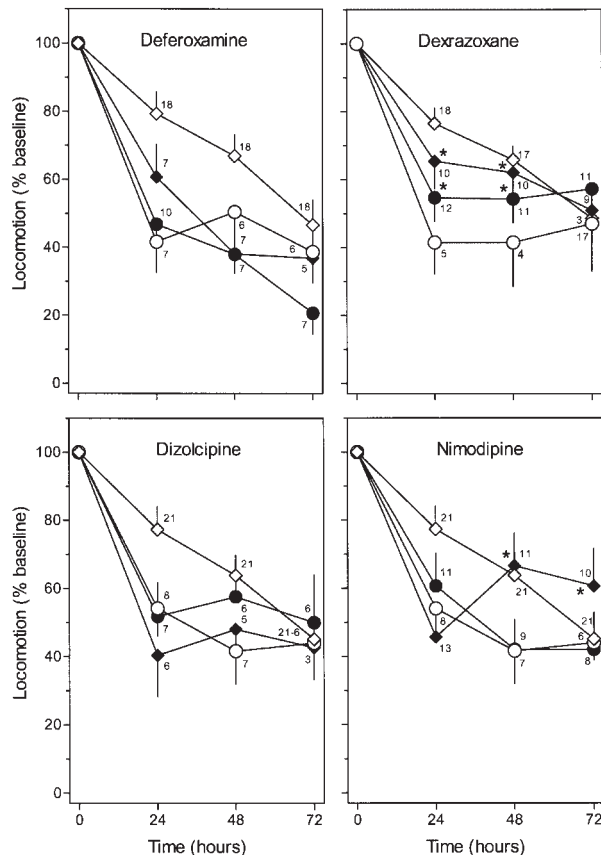

Fig. 3. Effect of dexrazoxane and reference neuroprotective agents on the hypolocomotion induced by sequential carotid artery sectioning in mice. Each panel shows spontaneous locomotion for sham-operated (open diamonds), vehicle-treated (open circles) and compound-treated animals. In each panel, solid circles and diamonds indicate low and high doses, respectively, of deferoxamine (32 and 128 mg/kg) dexrazoxane (32 and 128 mg/kg), dizolcipine (0.8 and 3.2 mg/kg), or nimodipine (0.4 and 1.6 mg/kg). Compounds or the corresponding vehicle were given i.p. 15 min after the second surgery. Locomotion was measured for 2-min periods with and Opto-Varimex animal activity meter. The abscissae represent time after compound administration; the ordinates, locomotor activity expressed as percent of the baseline counts. Values are the means $\pm$ S.E.M. for a variable number of animals (n $=$ 14–22 at the beginning of the experiment). *Significant increase ($P<0.05$) in locomotion as compared with the respective vehicle-treated group (ANOVA followed by Tukey's test).
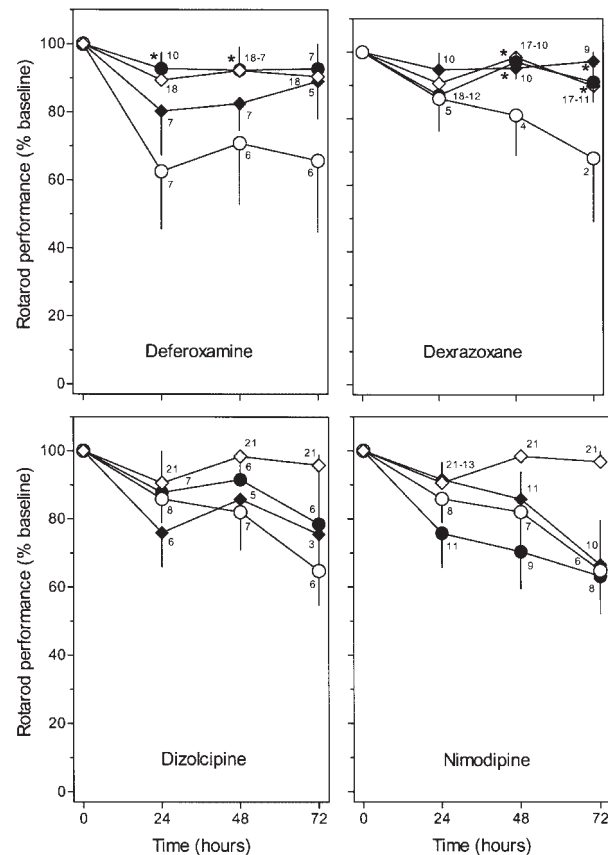
Fig. 4. Effect of dexrazoxane and reference neuroprotective agents on motor incoordination induced by sequential carotid artery sectioning in mice. Each panel shows rotarod performances for sham-operated (open diamonds), vehicle-treated (open circles) and compound-treated animals. In each panel, solid circles and diamonds indicate low and high doses, respectively, of deferoxamine (32 and 128 mg/kg) dexrazoxane (32 and 128 mg/kg), dizolcipine (0.8 and 3.2 mg/kg), or nimodipine (0.4 and 1.6 mg/kg). Compounds or the corresponding vehicle were given i.p. 15 min after the second surgery. The abscissae represent time after compound administration; the ordinates, rotarod performance expressed as percent of the baseline counts. Values are the means $\pm$ S.E.M. for a variable number of animals (n $=$ 14–22 at the beginning of the experiment). *Significant increase ($P<0.05$) in the time spent on the rotarod as compared with the respective vehicle-treated group (ANOVA followed by Tukey's test).

mice. The hypolocomotion produced by SCAS was also antagonized (48 and 72 h) by nimodipine (1.6 mg/kg). There were no differences in locomotion among animals receiving deferoxamine or dizolcipine and those injected with their vehicle.

Vehicle-treated mice stayed on the rotarod for a significantly shorter time than sham-operated animals (Fig. 4). The overall reduction in average time spent on the rotarod was 88, 90, and 84% at 24, 48, and 72 h, respectively, after the second surgery; these reductions were significantly different when compared with their respective control baseline. The time spent on the rotarod by animals treated with dexrazoxane (32 and 128 mg/kg) or deferoxamine (32 mg/kg) was significantly longer than that of vehicle-treated mice. In this test, dizolcipine and nimodipine had no detectable effects.

All operated animals lost body weight. Sham-operated mice lost almost 4 g, on average, as compared to their preoperative weight. However, they quickly regained their preoperative weight within a day or two. In contrast, vehicle-treated mice lost a significant proportion of their body weight. The mean difference between presurgical body weight and weights at 24, 48, and 72 h following the second surgery were 4.9 ± 1.2, 8.3 ± 0.9, and 9.1 ± 1.3 g, respectively. No significant differences were found between animals receiving vehicle injection and compound-treated animals (data not shown).

DISCUSSION

These experiments show that dexrazoxane possesses remarkable neuroprotective properties. In agreement with our previous findings [Rodriguez et al., 2000b], dexrazoxane substantially decreased the mortality produced by brain ischemia, i.e., it increased the survival rate of mice subjected to SCAS. The determined 24-, 48-, and 72-h overall survival rates for dexrazoxane (128 mg/kg) were 66, 66, and 60%, respectively, and represent a significant prolongation of life as compared with vehicle-treated animals (38, 28, and 21%, respectively). The log-rank test indicated that the median survival times of both dexrazoxane-treated groups were significantly different from the vehicle-treated group.

The major finding of this study indicates that dexrazoxane administration protected mice against the neurological impairment induced by SCAS. The determined 24-, 48-, and 72-h neurological scores for dexrazoxane (128 mg/kg) of 3.3, 3.5, and 4.0, respectively, as compared with the scores observed for its solvent-treated control group (6.5, 7.4, and 7.9, respectively), indicate that administration of dexrazoxane is beneficial for the neurological output of surviving animals. In addition, the protection provided by dexrazoxane treatment was also exerted on locomotion and motor coordination. These findings are critical because none of the three putative neuroprotective agents tested in this study reduced the mortality and/or consistently diminished neurological deficits. The present results and the fact that dexrazoxane is significantly more effective when given after brain ischemia than before it [Rodriguez et al., 2000b], show that this compound has very important neuroprotective properties in laboratory animals and clearly substantiate the idea that it may have therapeutic functional relevance in humans [Rodriguez et al., 2000b].

The question of the relative contribution of the described mechanism of action of dexrazoxane as a determinant for its neuroprotective properties [Rodriguez et al., 2000b] is raised again in this study. Dexrazoxane is a cardioprotective antioxidant that is clinically used to reduce the cardiotoxicity of the chemotherapeutic compound doxorubicin [Seifert et al., 1994]. The cardioprotective activity of this compound is thought to result primarily from the ability of the compound's hydrolysis products to chelate free or bound intracellular iron in the myocardium. This reduces the number of metal ions complexed with doxorubicine and, consequently, decreases the formation of superoxide radicals after redox recycling [Wiseman and Spencer, 1998]. The ability of dexrazoxane and its 1-ring-opened hydrolysis intermediates, and its 2-ring-opened hydrolysis product to remove iron from transferrin and ferritin, and from anthracycline-iron complexes has been demonstrated in vitro [Hasinoff and Kala, 1993; Buss and Hasinoff, 1993]. Consistent with these findings, in vivo studies have shown that while dexrazoxane inhibits doxorubicine-induced lipid peroxidation in mouse cardiac microsomal enzymes by 65%, its final hydrolysis products cause complete inhibition of lipid peroxidation [Hüsken et al., 1995]. If dexrazoxane protects cardiac cells from doxorubicine-induced damage by chelating intracellular iron, it could be possible that its protective activity against brain ischemia also involves iron-dependent free radical reactions, i.e., preventing iron catalyzed formation of $\cdot$OH. Whether this offers a satisfactory explanation for the neuroprotective action of dexrazoxane is not known. Alternatively, dexrazoxane might protect against ischemia via mechanisms different from its iron-chelating properties. The lack of activity observed with deferoxamine, a potent iron-chelator that strongly inhibits iron-dependent lipid peroxidation [Halliwell, 1989], raises this possibility. However, at present, we have no evidence to support such a hypothesis.

The lack of protective actions of deferoxamine, dizolcipine (NMDA receptor channel blocker), and nimodipine (calcium channel blocker) is notable. These compounds have been shown to provide effective neuroprotection against hypoxia-ischemia injury in laboratory animals [Mohamed et al., 1985; Park et al., 1988; Halliwell, 1989]. The discrepancy between the present findings and those reported by other authors might involve various factors. The most important is perhaps related to experimental procedures. For example, in many studies, infarct size is the most commonly used endpoint to assess the efficacy of potential therapies [Corbett and Nurse, 1998] although most evidence indicates that the capacity to reduce infarct size in laboratory animals does not correlate with neuroprotection in clinical studies [De Keyser et al., 1999]. In the present study, we used the combination of mortality and neurological outcome, the most important endpoints in clinical trails of neuroprotective agents, to determine the effects of brain ischemia and the degree of neuroprotection.

Another consideration is that most experimental studies that have shown neuroprotective activity for such compounds were conducted on healthy young to middle-aged animals. Differences in susceptibility to brain ischemia between young and aged animals have been clearly established [Yager et al., 1996; Fuentes-Vargas et al., 2002]. It is quite possible that treatments found effective in young animals might not necessarily be as effective in aged animals. Based on our previous experiments, this study was conducted in aged animals subjected to chronic forebrain hypoperfusion (comorbid condition) before the acute ischemic event. Another reason might be that most of the putative neuroprotective agents are more effective when given before the ischemic insult [Park et al., 1988; Wauquier et al., 1989]. Interestingly, dexrazoxane is more effective when given after the ischemic insult [Rodriguez et al., 2000b]. Finally, the possibility that our experimental model of ischemia is insensitive/inappropriate to reveal neuroprotection for this type of compounds cannot be discarded. However, this distinction might be potentially important because these compounds are not clinically active in humans.

On the other hand, as most compounds that reduce infarct size in laboratory animals have failed in humans, the importance of accurately assessing the neurological status of ischemic animals in studies on the efficacy of a given treatment is obvious. Estimation of functional capacity in animals by means of quantitative neurological evaluations provides a reliable measure to determine the course of impairment and the consequences of pharmacological manipulations. However, attempts to measure functional outcome in ischemic animals have been hampered by several problems: the range of neurological deficits, the great individual variations in severity and duration, as well as the fact that disability cannot be cited in dimensional data; hence, functional assessment requires the construction of arbitrary scoring scales. Despite these difficulties, efforts have been made to identify functional changes after the induction of brain ischemia, and a variety of neurological scoring systems and scales have been employed to measure the degree or severity of the neurological deficit [Nunn and Hodges, 1994; Corbett and Nurse, 1998; Block, 1999; De Vries et al., 2001]. However, no single measurement technique has yet proven to be universally acceptable, and most recently proposed protocols lack information regarding their capacity to detect pharmacological neuroprotection [Hunter et al., 2000].

This report proposes a simple procedure to determine functional output in mice after SCAS. The method presented has two complementary parts: (1) identification of the most consistent neurobehavioral alterations produced by SCAS, and (2) a general disability status scale. The neurological evaluation largely centers on motor function but also includes measurement of other brain functions. The disability status scale delineates six major progressive steps or grades of overall dysfunction attributable to brain ischemia. This scale is on a 0 to 10 basis, inasmuch as these represent the sum of neurologic impairments, it permits an equivalent grade for what is hoped to be an equivalent amount of the brain's functional damage, regardless of the structures involved. It should be mentioned that our NDSS represents only a rough adaptation of Kurtzke's scale used for rating neurological impairment in multiple sclerosis [Kurtzke, 1955, 1983].

Our strategy to produce brain ischemia and to evaluate functional outcome and compound effects is technically simple. However, it has some disadvantages: it is laborious, time consuming, requires large sample sizes, consistent experimental conditions, and time to train personnel to properly identify normal mice behavior and neurobehavioral alterations, and an equally long time to perform a complete quantitative study of a single compound. Therefore, it is not totally adequate for a primary screening of neuroprotective compounds. Additionally, it only provides a global, rough estimation of the functional status of ischemic animals, and does not provide information on which specific behaviors are improved or exacerbated following treatment. However, its major advantage could be its capacity to discriminate clinically relevant neuroprotection. Results of clinical trails with dexrazoxane in patients suffering stroke will respond to this important issue. But before clinical trials are attempted, further

research is needed to find the dosage required to produce optimal output benefits.

The finding that dexrazoxane administration yields a greater survival and better neurological outcome of ischemic animals strongly supports the idea that this compound has potentially useful neuroprotective activity. Importantly, none of the three tested putative neuroprotective agents produced equivalent protection. The present data support the usefulness of our procedure to evaluate the degree of neurological impairment after brain ischemia. Besides, it might be relevant to predict the effects of neuroprotective agents on the functionality of stroke patients.

ACKNOWLEDGMENTS

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DDR

Research Article

Age-Related Susceptibility to Brain Ischemia in Mice

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT   In this study, we evaluated the effect of age on survival and on neurological alterations produced by bilateral sequential common carotid artery (CCA) sectioning in mice. Male mice, 3–4 (young), 20–30 (adult), and 40–60 (aged) weeks old, underwent sequential CCA sectioning at an interval of 32 days. After the second surgery, mortality was registered continuously over a period of 24 h and thereafter every 24 h up to 3 days. Neurological assessment was performed 24 h after the second surgery. The influence of age on survival was analyzed using a log-rank test, and survival curves were generated by the Kaplan-Meier method. In mice with previous left CCA sectioning, sectioning of the contralateral artery induced a wide range of neurological alterations and a high mortality rate. Young and aged animals had a significantly lower ($P < 0.05$) survival rate as compared to adults (3, 6, 9, and 12 h after the second surgery), thus confirming that young and aged animals are, in fact, less resistant to brain ischemia. In contrast, the percentage of mice showing severe neurological alterations increased with age. This observation coincides with clinical evidence showing that elderly tend to have a worse outcome than younger patients do. These findings indicate that, besides morphological changes, studies of influence of age on the susceptibility to brain ischemia should include mortality and functional endpoints. Drug Dev. Res. 57:161–166, 2002.   © 2003 Wiley-Liss, Inc.

Key words: brain ischemia; susceptibility; survival; mice; age

INTRODUCTION

A variety of experimental methods have been developed to study the pathophysiology of ischemic stroke, and present knowledge on the intracellular events now regarded as primarily responsible for producing hypoxic-ischemic brain injury is mainly based on detailed studies in experimental animals. However, the value of such models in the discovery and development of neuroprotective agents has been questioned (Wiebers et al., 1990; Hunter et al., 1995). Most compounds that have been proven highly effective in reducing ischemic lesion size in experimental animals exhibit disappointing efficacy in clinical trials (De Keyser et al., 1999).

In view of the fact that it has not been possible to translate from animal models to the clinical situation, it has been claimed that the overall frustrating results of clinical trials are mainly due to methodological problems, since many claims of possible clinical efficacy have been made following protocols that are irrelevant to the clinical situation (Hunter et al., 1995;

De Keyser et al., 1999). For example, most experimental studies are conducted in young, healthy animals under rigorously controlled conditions (Ginsberg and Busto, 1989). In contrast, the typical stroke patient is elderly, with numerous risk factors and coexisting age-related diseases, and the etiology, location, and severity of the ischemic stroke are very heterogeneous (Futrell and Millikan, 1996). In experimental animals, infarct size is the most commonly used endpoint to determine the degree of injury and results of treatment. In contrast, survival and functional scores are typically used in humans to evaluate damage and efficacy of drugs. Therefore, it is necessary to continue the search for animal models that more closely approximate the clinical condition and are able to predict clinical efficacy of drugs.

We reported recently that in mice with previous left common carotid artery sectioning, the addition of right carotid sectioning, 32 days later, elicits profound neurological alterations, brain damage, and a high mortality rate (Rodriguez et al., 2000a). This new model of experimental ischemic stroke is essentially a model of global ischemia with one important difference. In most models of forebrain ischemia, occlusion of the carotid arteries is only transient, and both the onset of ischemia and its subsequent reversal are abrupt (Ginsberg and Busto, 1989). In our model, acute forebrain ischemia is produced by sectioning the right CCA in animals subjected to a significant reduction of blood supply by previous left CCA sectioning. We postulated that this model of global ischemia might be relevant to stroke in humans. It incorporates the idea of provoking an acute ischemic insult in animals subjected to chronic hypoperfusion (coexisting disease) and uses neurological and survival endpoints to determine the effects of brain ischemia and the degree of neuroprotection.

We have continued our efforts to refine this model to better reflect human stroke and to test its capacity to identify clinically effective neuroprotective drugs (Rodriguez et al., 2000b). Considering that ischemic stroke is typically a disorder of middle and late life (Bonita, 1992) and that the aging brain undergoes numerous biochemical (Phillis and Clough-Helfman, 1990; Funahashi et al., 1994), morphological (Kadar et al., 1990; West, 1993), and electrophysiological (Barnes, 1993; Geinisman et al., 1995) changes that could alter the pattern of vulnerability to cerebral ischemia and the susceptibility to drugs, it seemed pertinent to examine the effect of age on susceptibility to brain ischemia in this new model of forebrain ischemia.

In this study, we evaluated the effect of age on survival and neurological alterations produced by bilateral sequential common carotid artery sectioning in mice.

MATERIALS AND METHODS

Animals

Male mice of different ages from our own breeding facilities (CFW, initially obtained from Taconic Farms, Germantown, NY) were used in this study. Mice were classified as young (3–4 weeks old; weight, 18–26 g), adults (20–30 weeks old; weight, 36–53 g), and aged (40–60 weeks old; weight, 38–57 g). Animals were housed five per cage in a temperature-controlled room ($22 \pm 2°C$, relative humidity $55 \pm 3\%$) with an automatically timed cycle of 12/h light/darkness cycle (8 AM to 8 PM). Food (Purina Chow, St. Louis, MO) and water were available ad libitum. All animals were allowed to acclimate to the environmental conditions for at least one week prior to experimentation. Twelve hours before experiments, food was withheld with free access to water. This study was performed with permission of the local animal care committee under the provisions of the Declaration of Helsinki.

Procedure

Bilateral sequential common carotid artery sectioning was done as previously described (Rodriguez et al; 2000a). Briefly, mice were slightly anesthetized with sodium pentobarbital (20–30 mg/kg, ip) and the left common carotid artery (CCA) was exposed through a midline incision. The artery was sectioned between ligatures and the incision was closed with surgical thread. After surgery, mice were placed in a box warmed at 3°C until recovery (approximately 60 min) and then returned to their home cages. Thirty-two days later, groups of 19–24 animals were reanesthetized and the right CCA was sectioned (time zero). Three types of controls were used in these experiments, i.e., animals not subjected to anesthesia and surgery, anesthetized non-operated animals, and sham-operated mice undergoing anesthesia and full surgical procedures except for artery ligation and section.

Neurological Evaluation and Lethality

Mice were allowed to fully recuperate from anesthesia. Neurological evaluation, using the procedure described by Irwin for preclinical drug evaluation (1968), was performed 24 h after the second common carotid artery sectioning. An observer who had no knowledge of the respective age group conducted the neurological assessment. Based on previous observations (Rodriguez et al., 2000a), only the animals displaying a severe neurological syndrome, character-

ized by marked reduction of locomotor activity/ immobility, severe motor incoordination and muscular weakness, unresponsiveness to stimuli, circling to the left when held by the tail with feet on the floor, and failure to extend the left forepaw fully or its persistent flexion, were counted and used for statistical analysis. Lethality was noted continuously over a period of 24 h and then 48 and 72 h after the second CCA sectioning. The number of deaths was used to calculate survival curves.

Data Analysis

The difference in occurrence of severe neurological syndrome was tested with the Kruskal-Wallis analysis of variance followed by Dunn's multiple comparisons test. The survival curves were calculated by the Kaplan-Meier method (Motulsky, 1995) and compared using the log-rank test (2-tailed). The specific survival rate was analyzed at 3, 6, 12, 24, 48, and 72 h. Analysis was done using GraphPad Prism version 3.00 for Windows (GrapPad Software, San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Left CCA sectioning produced only mild, transient increase in locomotor activity, noted upon recovery from anesthesia. No differences were observed among each age group. No deaths were observed in anesthetized non-operated or in sham-operated mice of any age group (data not shown).

In mice with previous left CCA sectioning, sectioning of the contralateral artery gave rise to a wide range of neurological alterations and a high mortality rate. During the first 24-h follow-up, the number of deaths was 14/24 for the young group, 11/24 for the adult group, and 12/20 for the aged group. Figure 1 shows the corresponding Kaplan-Meier survival curves. The 24-h survival rate was 42% (CI = 22 to 63%), 54% (CI = 32 to 74%), and 40% (CI = 19 to 63%) for young, adult, and aged animals, respectively. As also can be seen in Figure 1, the survival rate was relatively similar in aged and young animals; in both cases, a significant number of deaths were observed during the early period (0–60 min) after sectioning the right CCA. In contrast, death in adult animals, except in two cases, was noted 6 h after the second surgery. The result of the log-rank test indicated significant group differences ($P < 0.05$) in survival between adult and young and between adult and aged animals at 3, 6, and 12 h after the second surgery. This difference was statistically significant in favor adult mice. No significant differences were detected between young and aged animals. In all groups, the survival rate

decreased gradually to 17% (young), 25% (adult), and 20% (aged) at the end of the experimental period (72 h). The difference between these three curves was not statistically significant at 24, 48, and 72 h.

The second surgery produced some early deaths in aged (4/24), and young (4/24) sham-operated animals. Except one case (1/24), all adult sham-operated mice survived for the entire experimental period (72 h). The 24-h survival rate was 83% (63 to 95%), 100% (86 to 100%), and 83% (63 to 95%) for young, adult, and aged mice, respectively. These differences were not statistically significant. When analyzed at the end of the experimental period (72 h) age-specific survival varied from 71% (aged) to 96% (adults). No deaths were observed in anesthetized non-operated animals.

The variety and severity of neurological alterations varied markedly among surviving mice of all age groups. Most animals showed hunched posture, lateralized body posture, decreased responsiveness to stimuli, passivity, slowness of movement, reduced locomotor activity, motor incoordination, circling, muscular hypotony, decreased muscular force, left front limb flexion, and respiratory difficulties. Less frequent alterations were rigid locomotion, irritability, tremor, seizures, rotation, and urinary incontinence. In a few mice, not significant neurological alterations were observed. Interestingly the percentage of mice showing severe neurological alterations, as defined in the previous section, clearly increased with age (Table 1). Statistical analysis revealed significant variation among medians ($P < 0.05$); however, there was not significant age-related trend; this was due to the small number of animals per group. None of the surviving sham-operated mice showed significant neurological abnormalities.

DISCUSSION

In most experimental studies, differences in susceptibility to brain ischemia between young and aged animals has been evaluated by counting the remaining number of healthy cells or calculating infarct volumes after the ischemia. The general finding has been that under similar experimental conditions, there is an age-related increase in cerebral infarct size (Davis et al., 1995; Futrell et al., 1991). However, recent experiments suggest that the correlation between brain damage and age is not linear. Yager et al. (1996) produced hemispheric global ischemia in rats aged from 1 week to 6 months and found that brain damage is most severe in 1- and 3-week-old animals followed by those that were 6 months. The 6- and 9-week-old groups had significantly less injury than the other three age groups. These results indicated that sensitivity to
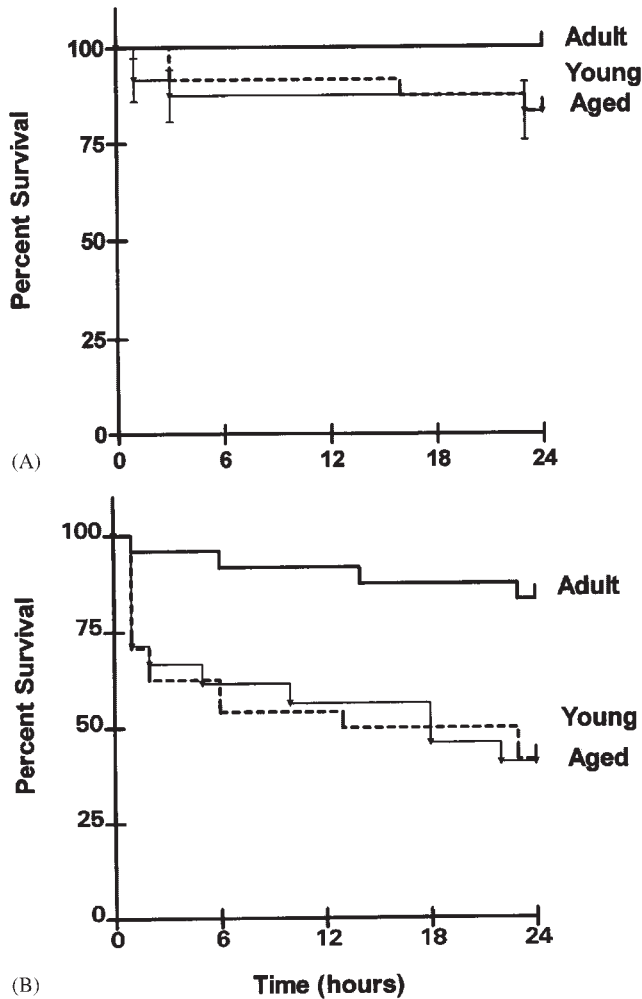
Fig. 1. Kaplan-Meier survival curves of mice of different age subjected to sham operation (**A**) or bilateral common carotid artery (CCA) sectioning (**B**). The 24-h survival of animals subjected to sequential CCA sectioning was 42% (CI, 22 to 63%), 54% (CI, 32 to 74%), and 40% (19 to 63%) for young, adult, and aged animals, respectively. Differences were statistically significant ($P < 0.05$) in favor of the adult animals (2-tailed log-rank test) at 3, 6, and 12 h after the second surgery. The three age groups subjected to CCA sectioning had significantly lower survival that their respective sham-operated control group ($P < 0.05$).

TABLE 1 Incidence of Severe Neurological Alterations in Young, Adult, and Aged Mice Subjected to Sham Operation or Sequential Common Carotid Artery Sectioning

Group	[a]Number of mice evaluated	Percentage of mice with severe neurological deficit
Young		
Sham	19	0
CCA sectioning	12	16.6
Adult		
Sham	19	0
CCA sectioning	12	25.0
Aged		
Sham	16	0
CCA sectioning	9	56.0

[a]Neurological evaluation was performed 24 h after the second surgery.

ing a linear correlation between neurologic deterioration due to brain ischemia and age. This observation coincides with clinical evidence showing that elderly patients are less resistant to an acute ischemic event (von Arbin et al., 1992; Dennis et al., 1993) and tend to have a worse outcome than younger patients (Nakayama et al., 1994).

The present study also makes it clear that assessing the influence of age on susceptibility solely on the basis of conventional histological procedures is not adequate, as has been suggested by others (Corbett and Nurse, 1998). This becomes particularly important in the light of accumulating evidence that neurological deficits and infarct size do not necessarily correlate (Corbett and Nurse, 1998). It is suggested, therefore, that measures of mortality and functional impairment should be considered in experimental stroke models used to detect clinically effective drugs. In fact, the combination of these two endpoints, mortality and functional outcome, are the most important endpoints in early clinical trials of neuroprotective agents. In conjunction with the present findings, measurement of disability is now being increasingly used to rate the status of animals after brain ischemia (Hunter et al., 2000).

The age-related differences in response to acute ischemia found in this study might have the following explanations. Firstly, the greater and earlier mortality observed in young and aged animals, as compared to adults, could be due in part to differences in metabolic responses to surgery. In humans, the increased rate and severity of perioperative complications of young and elderly patients are thought to be related to the reduced capacity of all major organ systems to meet the increased metabolic demands associated with surgical stress (Evers et al., 1994; Muravchick, 1998). In the elderly, there is also an increased risk of morbidity and mortality because of the high incidence

hypoxic-ischemia is greatest at either end of the age spectrum and least in the juvenile ages.

The results of the present study provide additional important information regarding the relationship of age to susceptibility of animals to brain ischemia. Using survival as one of the endpoint measures, our study confirms that young and aged animals are, in fact, less resistant to ischemia than their adult counterpart. In contrast, the percentage of mice exhibiting severe neurological deficits was higher in aged animals, followed by adult and younger animals; thus, suggest-

of coexisting age-related diseases. The lower survival rate of sham-operated young and aged animals compared to adults is consistent with these observations. Secondly, the greater incidence of severe functional disability and mortality observed in aged animals could be related to a reduced capacity of the aged cerebral vessels to rapidly redistribute blood from the vertebro-basilar circulation to carotid circulation and/or an inability to adequately regulate local cerebral blood flow in response to acute ischemia. The morphologic changes in the vasculature of the aging brain that could impair cerebral vascular autoregulation include reduced vascularity (Wilkinson et al., 1981), decreased capillary lumen diameter and capillary endothelial cells, increased microvascular tortuosity (Akima et al., 1986), and vascular thickening due to increased intramural collagen and other materials (Knox et al., 1980). Thirdly, there are biochemical/metabolic changes that occur with age. Among others, there is evidence of decreased intracellular ATP content (Joo et al., 1999), altered Ca^{2+} homeostasis and Ca^{2+} intracellular accumulation (Das and Ghosh, 1996), altered pH regulation (Roberts and Sick, 1996), poor recovery of ion homeostasis and synaptic transmission (Roberts and Chih, 1995), impairment of mitochondrial function (Yu et al., 1996), increased formation of free radicals and sensitivity to oxidative stress (Floyd, 1991; Joseph et al., 1996). All these changes suggest that with age the biological plasticity of the brain may be reduced to meet emergency conditions and could explain the worse ischemic outcome and higher mortality in older animals seen in this study. Precisely, why aging is associated to an increased vulnerability to ischemia is unclear but is a point that deserves more investigation.

Given the above findings, it is possible that treatments found effective in young animals would not necessarily be as effective in aged animals. Since clinical trials are done in elderly patients, this type of study suggests assessing the neuroprotective efficacy of drugs in models of ischemia using aged animals. The use of young animals to evaluate neuroprotection could be one of the factors explaining the lack of correlation between animal data and clinical trials.

The information derived from the present series of experiments warrants further efforts to continue the characterization of our model of global ischemia and to determine whether it is useful to predict the clinical effectiveness of drugs. In view of the wide range of behavioral and neurological alterations arising from the sequential common carotid artery sectioning, and considering that our present method is unable to detect subtle forms of neurological dysfunction, we are now in the process of constructing an index of functional disability to evaluate the consequences of brain ischemia and to guide the selection of promising candidates to take into clinical trials.

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Dexrazoxane-Induced Reduction in Mortality in Mice Subjected to Severe Forebrain Ischemia

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Strategy, Management and Health Policy			
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT   In this study we determined whether dexrazoxane reduces mortality in mice subjected to bilateral sequential common carotid artery sectioning. Under pentobarbital anesthesia, the left common carotid artery was ligated and sectioned. In one group of experiments, 32 days later mice were injected i.p. with dexrazoxane (16, 64, 256 mg/kg) 30 min before being reanesthetized to ligate and section the right common carotid artery. In the second group of animals dexrazoxane was given, at the same doses, 15 min after ligating and sectioning the right common carotid artery. Dexrazoxane significantly decreased the cumulated mortality rate compared with controls. When given 30 min before the second surgery dexrazoxane was active only at doses of 256 mg/kg. In contrast, when given 15 min after the second ischemic insult the protective effect of dexrazoxane was already evident at 16 mg/kg, increasing dose-dependently with an almost complete protection at 256 mg/kg during the first 24 h of observation; some degree of protection persisted up to day 4. One possible explanation for this striking difference in efficacy is that dexrazoxane passes through a compromised but not through an intact blood–brain barrier. We conclude that dexrazoxane has important neuroprotective properties against brain ischemia and that its clinical trial in stroke should be considered. Drug Dev. Res. 51:149–152, 2000.   © 2001 Wiley-Liss, Inc.

Key words: neuroprotection; dexrazoxane; brain ischemia; free radical production

INTRODUCTION

There is convincing evidence that cerebral ischemia and reperfusion are associated with the formation of several oxygen free species (ROS), including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl ($^\bullet OH$) [Ikeda and Long, 1990; Traystman et al., 1991; Phillis, 1994; Hall, 1997; Lipton, 1999]. Hydroxyl radicals are highly reactive oxidant agents and are thought to be the prime mediators of oxidative damage [Halliwell and Gutteridge, 1984; Halliwell, 1992; Hall, 1993]. In contrast, superoxide radicals are much less reactive, but have longer half-lives and can form hydroxyl radicals through a Haber-Weiss reaction. It is generally accepted that this reaction proceeds rapidly in the presence of transition metal ions (Fenton reaction), of which iron is the most important [Halliwell and Gutterridge, 1986; Minotti and Aust, 1989; Halliwell, 1992; Ryan and Aust, 1992].

Under nonpathological conditions, iron levels are tightly controlled and iron-catalyzed free radical reactions are kept minimal; however, in some situations the iron balance can be disturbed. Intracellular free iron increases significantly during ischemia, probably because of the accumulation of reducing equivalents that arise during ischemia and also due to ischemia-induced acidosis [Voogd et al., 1992]. This free iron is believed to catalyze the production of a pulse of hydroxyl radicals when oxygen tension is suddenly restored during reperfusion. A prominent effect of $^\bullet OH$ production is lipid peroxidation [Hall, 1993; Hall et al., 1993], but this radical also damages proteins, DNA, and other biomolecules.

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In favor of a major role of ROS in ischemia/reperfusion injury is the demonstration of the protective efficacy of free radical scavengers and lipid antioxidants in animal models of cerebral ischemia [Hall, 1997], and the role of iron in brain lipid peroxidation is supported by studies showing that iron-chelating agents, such as deferoxamine, lessen brain lipid peroxidation, decrease ischemic damage, and delay mortality in laboratory animals [Halliwell 1992; Aust and White, 1985; Halliwell and Gutteridge, 1986; Rosenthal et al., 1992].

Dexrazoxane is a powerful synthetic iron chelator that has been successfully used to reduce cardiac toxicity in patients receiving anthracycline-based chemotherapy for cancer [Seifert et al., 1994]. The cardioprotective activity of this drug is thought to result primarily from the ability of the drug's hydrolysis products to chelate free or bound intracellular iron in the myocardium. This reduces the number of metal ions complexed with anthracyclines and, consequently, decreases the formation of superoxide radicals after redox recycling [Wiseman and Spencer, 1998]. To our knowledge, the potential neuroprotective capacity of dexrazoxane has not been previously investigated.

The objective of this study was to establish whether dexrazoxane reduces the mortality in animals subjected to bilateral sequential common carotid artery (CCA) sectioning, a new model of global cerebral ischemia [Rodriguez et al., 2000].

MATERIALS AND METHODS
Animals

Male adult mice, 30–40 g, from our own breeding facilities (CFW, initially obtained from Taconic Farms, Germantown, NY) were used in this study. Animals were housed two per cage in a temperature-controlled room ($22 \pm 1°C$, relative humidity $55 \pm 3\%$) with an automatically timed 12/h light/darkness cycle (8 AM to 8 PM). Food (Purina Chow, St. Louis, MO) and water were available ad libitum. All animals were allowed to acclimate to the environmental conditions for at least 1 week prior to experimentation. Twelve hours before experiments, food was withheld with free access to water. This study was carried out with permission of the local animal care committee under the provisions of the Declaration of Helsinki.

Procedure

Bilateral sequential common carotid artery sectioning was done as previously described [Rodriguez et al., 2000]. Briefly, mice were anesthetized with sodium pentobarbital (47 mg/kg, i.p.) and, under an operating microscope, the left CCA was exposed through a midline incision. The artery was sectioned between ligatures and the incision was closed with surgical thread. Sham-operated mice underwent the same procedures except artery ligation and sectioning. After surgery, mice were placed in a box warmed to 37°C until recovery and then returned to their home cages. Thirty-two days later, groups of 11–14 animals were injected i.p. with dexrazoxane (16, 64, 256 mg/kg) 30 min before being reanesthetized to ligate and section the right CCA (time zero). In the second group of experiments, dexrazoxane, at the same doses, was given i.p. 15 min after the right common carotid artery was sectioned. The control groups received saline. Deaths were noted continuously over a period of 24 h and thereafter every 24 h up to 8 days.

Drugs

Dexrazoxane (Batch # TC96K08) was freshly prepared before use and dissolved in 0.9% saline; doses are expressed in terms of their salts. Injections were made i.p. in a volume of 0.1 ml/10 g body weight.

Data Analysis

We used the chi-square test with Yates' correction to compare mortality. Statistical significance was set at $P < 0.05$.

RESULTS

Most saline-treated animals died within 60 min after the second surgery and the cumulated mortality rate was maximal or neared maximal 24 h after surgery (Fig. 1). No deaths were observed in sham-operated mice.

Dexrazoxane significantly decreased the cumulated mortality rate compared with controls (i.e., no treatment with dexrazoxane). When given 30 min before the second surgery, dexrazoxane was active only at doses of 256 mg/kg. In contrast, when given 15 min after the second surgery the protective effect of dexrazoxane was evident at 16 mg/kg and increased dose-dependently (Fig. 1). In this case, the cumulated mortality rates at 24 h were 100% in the saline-treated group and 20% in animals receiving 256 mg/kg, indicating that dexrazoxane reduced mortality by 80%. The protection by the highest dose of dexrazoxane apparently persisted up to day 4. The majority of drug-treated animals surviving more than 24 h showed decreased locomotion, motor incoordination, and decreased body weight; some delayed deaths were apparently caused by physical incapacity to look for food and water. In a few mice, there was not evidence of behavioral alterations. In this study no attempts were made to grade the effect of drug treatment on neurological alterations.

DISCUSSION

We recently reported [Rodriguez et al., 2000] that in mice with previous left CCA sectioning the addition of right CCA sectioning leads to characteristic neuronal
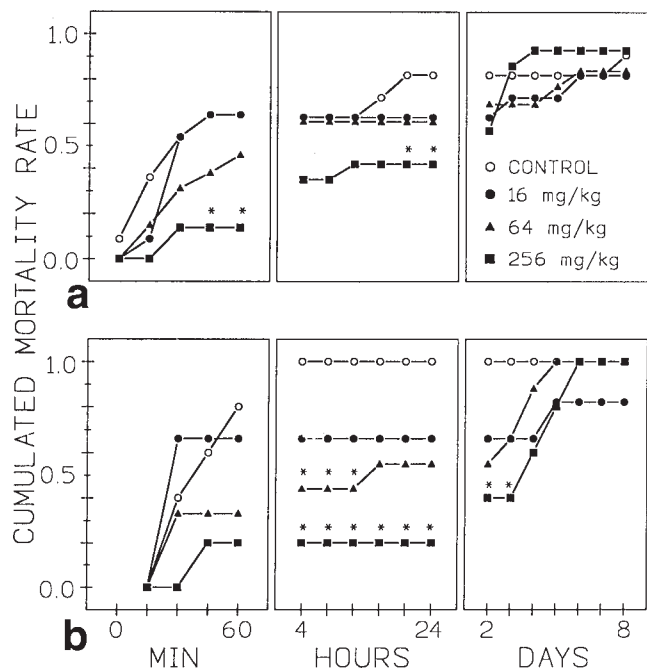
Fig. 1. Time course of influence of dexrazoxane on cumulated mortality rate of mice. Dexrazoxane was given i.p. at the indicated doses 30 min before (**a**) or 15 min after (**b**) the second common carotid artery ligation and sectioning (time zero). Deaths were noted continuously for the first 24 h and thereafter every 24 h up to 8 days. All values are given as the index of cumulated mortality rate for 11–14 mice. *$P < 0.05$, chi-square test with Yates' correction.

damage, a wide range of neurological alterations, and a consistent pattern of mortality. This new model of experimental ischemic stroke is essentially a model of global ischemia, with one important difference. In most commonly used models of cerebral ischemia, occlusion of the arteries is only transient, and both the onset of ischemia and its subsequent reversal are abrupt. In our model, there is a 32-day interval between sectioning of the common carotid arteries. We chose this interval to induce a background chronic state of cerebral hypoperfusion before the second ischemic insult. Using this model we found that some anti-ischemic agents substantially reduce the severity of neurological alterations and the cumulated mortality rate. We postulated that these endpoints could be used in the quantitative evaluation of the neuroprotective efficacy of drugs.

The present study demonstrates that dexrazoxane substantially reduces the cumulated mortality rate in mice subjected to bilateral sequential common carotid artery section. But the most important finding of these experiments is that dexrazoxane is considerably more effective when given after the second ischemic insult. In this case, reduction in mortality was clearly dose-dependent and the protective activity was apparent even at 16 mg/kg.

The wide gap in cumulated mortality between dexrazoxane-treated and saline-treated mice during the first 24 h (Fig. 1) indicates that the protective effect lasted at least throughout this period. In contrast, when given before (30 min) the second ischemic insult, the protection was less marked and evident only at 256 mg/kg. One possible explanation for this striking difference in efficacy is that dexrazoxane passes through a compromised blood–brain barrier but not through an intact blood–brain barrier. Findings by others support this idea. Disposition studies in laboratory animals indicate that penetration of dexrazoxane to the central nervous system is low. In rats, i.p. doses of 100 mg/kg of ICRF-159, the racemate of the (+)-enantiomer ICRF-187 (dexrazoxane), reached maximum levels in the CSF of approximately 1 μ/ml, equivalent to 10% of the plasma concentration [Grieg et al., 1982]; similar results have been reported using rhesus monkeys [Von Hoff et al., 1980]. The pharmacokinetics of dexrazoxane has also been studied in cancer patients and available information indicates that this drug does not cross the blood–brain barrier to a clinically significant extent [Earhart et al., 1982]. There is also evidence that forebrain ischemia in the rat produces an immediate, transient opening of the blood–brain barrier in several brain areas [Preston et al., 1993]. Thus, it is quite possible that the greater efficacy of dexrazoxane when given after the ischemic insult is due to its greater penetration to the ischemic area. In any case, the clinical significance of a drug highly active when given after an ischemic event cannot be disregarded.

The protective mechanisms and pharmacological properties of dexrazoxane that are responsible for its effect on mortality have not been clarified in this study. The ability of dexrazoxane and its 1-ring-opened hydrolysis intermediates and its 2-ring-opened hydrolysis product to remove iron from transferrin and ferritin, and from anthracycline-iron complexes, has been demonstrated in vitro [Hasinoff and Kala, 1993; Buss and Hasinoff, 1993]. Consistent with these findings, in vivo studies have shown that while dexrazoxane inhibits doxorubicin-induced lipid peroxidation in mouse cardiac microsomal enzymes by 65%, its final hydrolysis product causes complete inhibition of lipid peroxidation [Hüsken et al., 1995]. If dexrazoxane protects cardiac cells from doxorubicin-induced damage by chelating intracellular iron, it could be possible that its protective activity against ischemia also involves iron-dependent free radical reactions, i.e., preventing iron catalyzed formation of $^\bullet$OH. The precise mechanism by which dexrazoxane reduces mortality of ischemic animals has yet to be determined.

It is important to mention that dexrazoxane also reduces the severity of neurological alterations; however, in the present set of experiments we did not attempt to quantify this effect. In view of the large individual varia-

tions and because the wide range of behavioral, neurological, and autonomic alterations provoked by the second common carotid artery section, we decided to develop strategies, standards, and criteria to design an index of functional disability that encompasses the range of neurological deficits provoked by ischemia. Studies are ongoing to determine whether our scale can effectively rate neurological status of ischemic animals and discriminate protection and functional outcome of drug-treated mice.

To our knowledge, this is the first report on the neuroprotective properties of dexrazoxane and our findings clearly indicate the relevance of clinical trials with dexrazoxane in patients suffering stroke.

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