ESTUDIO DE LAS PROPIEDADES ANTIINFLAMATORIAS DE LA HEPARINA NO FRACCIONADA EN LA ISQUEMIA CEREBRAL

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CERTIFICAMOS que la memoria titulada “ESTUDIO DE LAS PROPIEDADES ANTIINFLAMATORIAS DE LA HEPARINA NO FRACCIONADA EN LA ISQUEMIA CEREBRAL”, presentada por Álvaro Cervera Álvarez, ha sido realizada bajo nuestra dirección y consideramos que reúne las condiciones necesarias para ser defendida ante el Tribunal correspondiente para optar al Grado de Doctor en Medicina y Cirugía.

Dr. Ángel Chamorro Sánchez Dra. Anna Maria Planas Obradors

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A Yolanda

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I. INTRODUCCIÓN

La heparina no fraccionada (HNF) se ha utilizado en la práctica clínica desde hace más de 50 años como fármaco anticoagulante. Un estudiante de la Universidad Johns Hopkins, McLean, descubrió una sustancia que aumentaba el tiempo de coagulación a la que llamó heparina debido a que era abundante en el hígado (McLean, 1959). Aunque la mayor parte de los trabajos sobre la estructura y función de la heparina se han concentrado en el estudio de las interacciones responsables de su papel en la inhibición de la coagulación sanguínea, en los últimos años la investigación se ha ampliado incluyendo importantes aplicaciones antiinflamatorias (revisado por Tyrrel et al, 1999).

Heparina no fraccionada: estructura y función.

La HNF y su polímero relacionado el heparán sulfato son miembros de una familia de polisacáridos denominada glucosaminoglicanos (Höök et al, 1984). Debido a las diferencias en la composición y la extensión de sulfatación, la HNF está más cargada que el heparán sulfato. Esto proporciona una mayor actividad biológica a la HNF, debido a la presencia de grupos iduronato. El resultado del proceso biosintético de la HNF es una polímero heterogéneo, con gran carga, compuesto principalmente de residuos de glucosamina N,6-disulfato e iduronato 2-sulfato.

La HNF y el heparán sulfato se sintetizan en el retículo endoplasmático como proteoglucanos. La HNF se sintetiza exclusivamente en los mastocitos del pulmón, intestino e hígado unida a la proteína serglicina (Toledo et al, 1977). El hecho de que los tejidos que contienen HNF sean aquellos en contacto directo con el ambiente sugiere un papel de la HNF en la defensa del huésped (Nader et al, 1989). La HNF comercial se obtiene de mucosa intestinal porcina o bovina.

La acción anticoagulante de la HNF reside en su habilidad para potenciar la actividad de la antitrombina (Bourin et al, 1993). La HNF también interacciona con otro inhibidor de la serin proteasa, el cofactor de la heparina II, para potenciar la inhibición de la trombina. La HNF se une a los sitios de lisina de la antitrombina, produciendo un cambio conformacional en el centro reactivo de la arginina, lo que convierte a la antitrombina de un lento y progresivo inhibidor de la trombina a un inhibidor muy rápido (Hirsh y Raschke, 2004). La unión a la antitrombina tiene lugar a través de una unidad de glucosamina única contenida en una secuencia pentasacárida (Choay et al, 1981).

La HNF es muy heterogénea, siendo su peso molecular entre 3.000 y 30.000. Tan sólo un tercio de la dosis administrada se une a la antitrombina (Lam et al, 1976). El complejo HNF-antitrombina inactiva a la trombina y los factores Xa, IXa, XIa y XIIa (Hirsh y Raschke, 2004). Los factores más sensibles son la trombina y el Xa, siendo el primero 10 veces más sensible a la inhibición. La capacidad de las moléculas de heparina para inactivar la trombina y otros factores de la coagulación activados es dependiente de la longitud de su cadena, según se ha podido demostrar utilizando fracciones de heparina de bajo peso molecular (HBPM). Por el contrario, la inactivación del factor Xa requiere tan sólo la presencia del pentasacárido de alta afinidad. Al inactivar la trombina, la HNF previene la formación de fibrina y la activación de plaquetas y de los factores de coagulación V y VIII dependientes de la trombina (Beguin et al, 1988).

Evidencia clínica de la anticoagulación en la fase aguda del ictus isquémico.

Los anticoagulantes se administran a los pacientes con ictus isquémico agudo con la intención de prevenir la progresión del trombo, facilitar la circulación colateral, dificultar el desarrollo de la recurrencia isquémica temprana y prevenir la trombosis venosa profunda y el embolismo pulmonar (Sherman et al, 1995).

Varios ensayos clínicos aleatorizados han evaluado la eficacia y la seguridad de la administración de una dosis fija de HNF y de las HBPM en pacientes con ictus isquémico agudo. Una revisión sistemática de 23.427 pacientes con ictus anticoagulados durante las primeras dos semanas del inicio de los síntomas demostró que el tratamiento se asociaba con un descenso de 9 recurrencias isquémicas por cada 1000 pacientes tratados (Gubitz et al, 2004). Sin embargo, este beneficio se veía superado por un aumento similar en el número de hemorragias intracraneales sintomáticas (Sandercock et al, 1993; Gubitz et al, 2004). A pesar de las teóricas desventajas biológicas de la HNF, la mayor tasa de hemorragia se dio entre las HBPM.

A pesar de no haber una prueba convincente de su eficacia terapéutica los médicos han administrado HNF a sus pacientes con ictus desde hace más de 50 años y HBPM desde al menos dos décadas. Sin embargo, los expertos en la medicina basada en la evidencia advierten que los anticoagulantes no deberían ser utilizados a dosis terapéuticas en pacientes con ictus isquémico agudo, ya que no ofrecieron beneficios en los ensayos

clínicos aleatorizados o en los metaanálisis (Gubitz et al, 2004). A pesar de todo, algunas guías terapéuticas recomiendan la anticoagulación inmediata en los pacientes con mayor riesgo de ictus recurrente, mientras que en la mayoría se prefiere retrasar la anticoagulación varios días en los pacientes con un bajo riesgo de recurrencia temprana (Sherman et al, 1995; Mohr et al, 1997). Otros autores prefieren la consideración individual caso a caso, dependiendo del mecanismo etiológico del ictus, la localización del vaso afecto y la extensión del proceso aterosclerótico (Caplan, 2003). Los investigadores del grupo Cochrane, descartan el uso de cualquier tipo de anticoagulante en el ictus isquémico agudo (Gubitz et al, 2004).

El International Stroke Trial (IST) es el estudio más importante en el que se ha evaluado el papel de la HNF en pacientes con un ictus isquémico establecido (International Stroke Trial Collaborative Group, 1997). Los pacientes a los que se les asignó un tratamiento con HNF (12.500 UI, dos veces al día) presentaban menos recurrencias isquémicas en los primeros 14 días que los pacientes no tratados con HNF (2.9% contra 3.8%). Sin embargo, este beneficio se compensaba por un aumento similar en los ictus hemorrágicos (1.2% contra 0.4%). Por lo tanto, no existió una diferencia significativa entre los tratamientos en mortalidad o recurrencia isquémica. Así, la incidencia combinada de hemorragia intracraneal, ictus recurrente y embolismo pulmonar fue 3.4% en pacientes tratados con aspirina y dosis baja de HNF, 4.4% en pacientes tratados tan sólo con aspirina y 4.7% en los pacientes tratados con dosis baja de HNF.

El Fraxiparine in Stroke Study (FISS) demostró que la HBPM nadroparina era mejor que el placebo al mejorar el pronóstico tras el ictus isquémico (Kay et al, 1995). Sin embargo, este hallazgo positivo no pudo ser replicado en el estudio FISS bis, realizado con pacientes europeos (Hommel et al, 1998).

En el estudio TOAST, Trial of ORG 10172 in Acute Stroke Treatment (The Publications Committee for the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators, 1998), un tratamiento de 7 días con danaparoide o placebo se administró en dosis única durante las primeras 24 horas del ictus. A los 3 meses un 75.2% de pacientes tratados con danaparoide y un 73.7% de los tratados con placebo tuvieron un pronóstico favorable (diferencia sin significación estadística). A los 7 días un 33.1% de los pacientes tratados y un 27.8% de los que recibieron placebo tuvieron un curso clínico muy favorable (P=0.01). Catorce pacientes tratados y sólo 4 de los que recibieron placebo sufrieron una

hemorragia intracraneal grave (P=0.05).

El estudio HAEST, Heparin in Acute Embolic Stroke Trial, comparaba el uso de otra HBPM, la dalteparina, (100 UI/kg subcutánea cada 12 horas) con aspirina (160 mg/día) en 449 pacientes con ictus isquémico agudo y fibrilación auricular (Berge et al, 2000). La frecuencia del ictus isquémico recurrente durante las primeras dos semanas fue 8.5% en los pacientes tratados con dalteparina y 7.5% en los asignados a aspirina (OR 1.13, 95% IC 0.57-2-24). No se encontraron diferencias significativas en el pronóstico funcional o muerte a los 14 días o a los 3 meses. Pero sí que aparecieron más hemorragias cerebrales sintomáticas en el grupo tratado con dalteparina (2.6% contra 1.7%).

El estudio TAIST, Tinzaparin in Acute Ischemic Stroke, comparó el tratamiento con la HBPM tinzaparina a dos dosis (175 anti-Xa UI/kg/día y 100 anti-Xa UI/kg/día) y aspirina (300 mg/día) (Bath et al, 2001). La proporción de pacientes independientes a los 6 meses fue 41.5% en el grupo de dosis alta de tinzaparina, 42.4% en el de dosis baja y 42.5% en el grupo de tratados con aspirina. La hemorragia intracraneal sintomática fue significativamente mayor en el grupo de dosis alta de tinzaparina.

En el estudio TOPAS, Therapy Of Patients with Acute Stroke, se administró la HBPM certoparina a 404 pacientes en 4 dosis diferentes durante las primeras 12 horas del inicio de los síntomas del ictus (Diener et al, 2001). La proporción de pacientes con un índice de Barthel mayor de 90 puntos no fue diferente entre los distintos grupos de tratamiento. En el seguimiento a los 6 meses el porcentaje de pacientes con ictus recurrentes fue similar en todos los grupos. Los investigadores concluyeron que el aumento de dosis de certoparina hasta las 8000 anti-Xa UI cada 12 horas no mejora el pronóstico funcional de los pacientes con ictus isquémico y que el sangrado grave tendía a ser más frecuente en el grupo con una dosis mayor.

Revisión crítica de los ensayos clínicos de anticoagulación

El estudio IST recomendaba realizar un TC craneal en el momento de la aleatorización para descartar una hemorragia cerebral. Sin embargo, 5600 pacientes obtuvieron su primera neuroimagen tras el inicio del tratamiento, por lo que es probable que se hubieran incluido erróneamente en el estudio unas 500 hemorragias intracraneales (Chamorro,

1999). Otra limitación importante del estudio IST fue la falta de monitorización de los efectos biológicos de la HNF. La razón que adujeron los autores fue que aun la dosis más alta de HNF asignada en el estudio (25.000 UI) era considerada segura en los ensayos clínicos conducidos previamente en pacientes con trombosis venosa profunda.

Con la excepción del estudio TOAST, en el que se ajustó la velocidad de la infusión del tratamiento para alcanzar el efecto biológico pretendido, el resto de estudios evaluaron los efectos de dosis diferentes de HNF o HBPM sin ajustar la dosis.

Otra limitación fundamental de muchos de estos estudios es el gran retraso en el inicio del tratamiento, entre 24 y 48 horas, una ventana de oportunidad terapéutica muy por debajo de los límites más optimistas de la penumbra isquémica. Mientras que este retraso en el tratamiento parece ser apropiado para disminuir el riesgo de embolismo pulmonar o trombosis venosa profunda, es menos probable que permita una adecuada protección neuronal, ya que la mayoría de eventos responsables de la muerte neuronal tiene lugar en una fase mucho más temprana (Chamorro, 1999). Además, en el estudio IST la HNF se administró de forma subcutánea y, por lo menos, se deben de añadir 24 horas para que se alcancen niveles de anticoagulación estables (Hirsh et al, 2001).

A pesar de estos inconvenientes, tras la finalización de estos estudios se llegó al convencimiento de que las heparinas y los heparinoides tienen un papel nulo o muy limitado en la prevención del ictus recurrente temprano y en la mejoría del pronóstico funcional (Gubitz et al, 2004). Sin embargo, estas conclusiones podrían ser prematuras, especialmente si prestamos atención a otros estudios que demuestran que los mecanismos inflamatorios que acontecen en la isquemia cerebral podrían ser modulados por la administración muy temprana de algunos anticoagulantes (Yanaka et al, 1996; Chamorro, 2001).

El riesgo de conversión hemorrágica aumenta por el uso de anticoagulantes en el ictus isquémico agudo (Drake et al, 1983). Según un metaanálisis (Gubitz et al, 2004), el uso de anticoagulantes se estimó que aumentaba el riesgo de conversión hemorrágica sintomática con una razón de odds (odds ratio, OR) de 2.52 (IC 95% 1.92-3.32). Hay indicios de que el sangrado es más frecuente cuando las pruebas de coagulación se prolongan excesivamente (Levine et al, 2004). En series clínicas de pacientes con ictus tratados con HNF la hemorragia intracerebral, tanto sintomática como asintomática,

ocurrió más frecuentemente después de una excesiva prolongación del tiempo de tromboplastina parcial activada (TTPA) (Chamorro et al, 1995). En pacientes con ictus isquémicos grandes la pregunta crucial a plantearse no es la seguridad de la anticoagulación, sino resolver si hay suficiente tejido cerebral salvable remanente tras el evento inicial como para justificar un riesgo de sangrado, aunque sea bajo. Si la respuesta es afirmativa, el riesgo-beneficio de la HNF se ha de incrementar a través de una administración juiciosa del fármaco.

Para muchos médicos el riesgo individual que tiene un paciente de presentar una recurrencia precoz es un elemento clave en la evaluación de la necesidad de prescribir anticoagulantes. Algunos expertos recomiendan un retraso en el tratamiento de unos días antes de la iniciación de la anticoagulación para minimizar el riesgo de sangrado intracraneal (Mohr et al, 1997). Así como estudios más antiguos hablaban de un riesgo de recurrencia temprana de un 10-20% durante los primeros 10 días del evento isquémico (Cerebral Embolism Study Group, 1987; Koller, 1982), los estudios más recientes en los que se incluían pacientes tratados con placebo, proporcionaban porcentajes menores de recurrencias isquémicas tempranas. Algunos autores (Lodder et al, 1988) estiman un riesgo de recurrencia superior al 8% en pacientes con ictus cardioembólico, aunque otros (Halperin et al, 1988) estiman un riesgo de entre un 15 y un 20% durante el primer año.

La evidencia a favor o en contra de la administración de una dosis ajustada de HNF en pacientes con ictus isquémico es escasa (Cerebral Embolism Study Group, 1983; Duke et al, 1986; Dobkin, 1983). Los datos disponibles sobre la anticoagulación “inmediata” son también inadecuados, especialmente si tenemos en cuenta que un retraso de 2 semanas sobrepasa el concepto de la inmediatez en el tratamiento del ictus isquémico. Además, pocos estudios enfatizan la relevancia de la dosis ajustada de HNF (Chamorro et al, 1995) y la rapidez en el inicio del tratamiento (Chamorro et al, 1999). Así, en dos estudios clínicos de pacientes tratados con HNF en la fase aguda del ictus isquémico la recuperación fue mayor en los pacientes tratados durante las primeras 6 horas del inicio de los síntomas y la recurrencia del ictus y el sangrado grave se asociaron con resultados anormales de las pruebas de coagulación (Chamorro et al, 1995; Chamorro et al, 1999).

En el estudio del Cerebral Embolism Study Group (1983) se aleatorizaron pacientes con ictus isquémico de origen cardioembólico de menos de 48 horas de evolución. La falta de complicaciones y la tendencia clara, en opinión de los autores, hacia la eficacia de la HNF

hicieron terminar prematuramente el estudio cuando tan sólo habían sido tratados 45 pacientes.

En cuanto al tema del deterioro clínico precoz, Duke et al (1986) estudiaron 225 pacientes estables durante al menos 48 horas después del inicio de los síntomas para evaluar si la HNF era más efectiva que el placebo en la prevención de la progresión del ictus que ocurría después del segundo día del ictus. Los resultados de este estudio fueron decepcionantes porque la tasa de progresión en los pacientes anticoagulados fue del 17%, mientras que en los tratados con placebos fue del 19.5%. Sin embargo, una limitación importante del estudio es el retraso tan largo hasta el inicio del tratamiento. De hecho, el ritmo de progresión del ictus tal como se vio en los ensayos clínicos en pacientes tratados con placebo es muy alto en las primeras horas del inicio de los síntomas. Así, en el estudio ECASS el 23% de los pacientes empeoraban de sus síntomas en las primeras 8 horas y el 37.5% en las 30 horas (Davalos et al, 1999). Estos datos sugieren que en el trabajo de Duke y colaboradores no se seleccionó el grupo de pacientes ideal para estudiar la prevención de la progresión del ictus, ya que presumiblemente los mejores candidatos ya se excluyeron del estudio, debido a que los pacientes debían permanecer estables durante las primeras 48 horas para poder ser incluidos. En otro estudio pequeño no aleatorizado, Haley et al (1988) no pudieron parar la progresión de los síntomas de empeoramiento en 36 pacientes consecutivos tratados con HNF ajustada para mantener el TTPA entre 1.5 y 2 veces la del control.

La inflamación es un mecanismo involucrado en el daño cerebral tras la isquemia.

La lesión cerebral isquémica es el resultado de una secuencia compleja de eventos fisiopatológicos que evolucionan en el tiempo y en el espacio (Dirnagl et al, 1999). Los mecanismos más importantes de esta cascada son la excitotoxicidad, las despolarizaciones periinfarto, la inflamación y la muerte celular programada.

Es bien conocida la contribución de procesos inflamatorios en la progresión de la isquemia cerebral (Kochanek et al, 1992; Feuerstein et al, 1994; Chamorro et al, 2006). La respuesta inflamatoria que acontece en la fase aguda del ictus es un mecanismo importante de muerte neuronal, sobre todo cuando existe reperfusión. Además, ocurre rápidamente, ya que las células y moléculas que intervienen en ella, citocinas, factores de

adhesión y leucocitos, se activan durante la primera hora de la isquemia, existiendo un pico de respuesta entre las 6 y las 12 horas (Feuerstein et al, 1995).

La activación de sistemas de segundo mensajero dependientes del calcio, el aumento de radicales libres de oxígeno, así como la hipoxia misma, desencadenan la expresión de varios genes proinflamatorios al inducir la síntesis de factores de transcripción, como el factor nuclear-κB, el factor inducible por la hipoxia-1 (Ruscher et al, 1998), el factor regulador del interferón 1 y la STAT3. Así, mediadores de la inflamación, como el factor activador de las paquetas, el factor de necrosis tumoral α (TNF-α) y la interleucina (IL)-1β son producidos por las células cerebrales lesionadas (Rothwell et al, 1995). Posteriormente se induce la expresión de moléculas de adhesión en la superficie de las células endoteliales, incluyendo la molécula de adhesión intercelular-1 (ICAM-1), las P- y las E-selectinas (Zhang et al, 1998; Haring et al, 1996; Lindsberg et al, 1996). Las moléculas de adhesión interaccionan con sus receptores complementarios en los neutrófilos. Los neutrófilos, a su vez, se adhieren al endotelio, cruzan la pared vascular y entran en el parénquima cerebral (Dirnagl et al, 1999). Los macrófagos y los monocitos siguen a los neutrófilos, y migran al cerebro isquémico donde serán las células predominantes a partir de los 5-7 días de isquemia. En el tejido cerebral dañado se producen citocinas, como la IL-8 y la proteína quimioatractante monocitaria-1, que guiarán la migración de las células inflamatorias procedentes del torrente sanguíneo hacia su diana.

Los leucocitos polimorfonucleares son los participantes más precoces en la respuesta microvascular cerebral a la isquemia focal, apareciendo en el tejido cerebral isquémico a partir de los 30 minutos de isquemia (del Zoppo et al, 1991; Garcia et al, 1994). El movimiento inicial de estas células inflamatorias no residentes dentro del sistema nervioso central requiere la aparición de receptores de adhesión leucocitaria (P-selectina, ICAM-1 y E-selectina) en el endotelio microvascular y sus contrareceptores leucocitarios (Mac-1). La mayor parte de las células del cerebro isquémico producen IL-1β y TNF-α. La exposición de las células endoteliales a esas citocinas provoca el aumento de expresión de ICAM-1 y E-selectina. La activación leucocitaria también puede producir oclusión microvascular y fenómeno de no-reflujo (del Zoppo et al, 1991).

La ICAM-1 es necesaria para la adhesión de células mononucleares y granulocitos al endotelio vascular. Su expresión se induce en los microvasos en áreas de infarto y los

niveles de la molécula soluble aumentan en sangre periférica tras el ictus (Shyu et al, 1997). La VCAM-1 es una molécula involucrada en la adhesión y trasmigración monocitaria (Osborn et al, 1989; Fassbender et al, 1995). La VCAM-1 es un factor de adhesión que se expresa de forma intensa por los astrocitos y las células endoteliales del tejido infartado en el córtex cerebral humano (Blann et al, 1999). La VCAM-1 induce la expresión de factor tisular, que es el iniciador de la cascada de la coagulación in vivo (McGilvray et al, 1997). La ICAM-1 sérica tiene un pico a las 24 horas del inicio de la isquemia, mientras que la VCAM-1 sérica alcanza su máximo a los 5 días y, por el contrario, la E-selectina sérica disminuye durante los primeros 5 días (Bitsch et al, 1998).

La IL-10 y la IL-4 son moléculas antiinflamatorias, principalmente secretadas por linfocitos y monocitos, que bloquean acciones proinflamatorias (Tedgui et al, 2001). En pacientes con ictus isquémico agudo se ha observado un aumento en las concentraciones de IL-10 en plasma, líquido cefalorraquídeo y células mononucleares circulantes (Perini et al, 2001). En un estudio se asociaron los niveles más bajos de IL-10 medidos en las primeras 24 horas de isquemia con el deterioro precoz de los síntomas neurológicos (Vila et al, 2003). Esta relación fue independiente de los predictores ya conocidos de empeoramiento clínico. La IL-10 inhibe la síntesis de IL-6 y TNF-α por parte de los monocitos/macrófagos al bloquear la transcripción genética y reducir la liberación de ICAM-1 y metaloproteinasas de matriz extracelular. Además la administración de IL-10 tiene efectos neuroprotectores en un modelo de isquemia cerebral focal de rata (Spera et al, 1998).

Las proteínas de choque térmico o de calor (Heat-shock proteins, HSP) son inducidas, en parte, por proteínas desnaturalizadas producidas durante el choque térmico, la isquemia y otro tipo de condiciones de estrés celular (Sharp et al, 1999). Las HSP funcionan como chaperonas que se unen a otras proteínas y regulan su conformación, regulan los movimientos de las proteínas a través de las membranas o de los orgánulos o regulan la disponibilidad de un receptor o la actividad de un enzima. Algunas de las HSP conocidas son la ubiquitina, HSP-10, HSP-27, HSP-32 o hemooxigenasa-1 (HO-1), HSP-47, HSP-60, HSC 70, HSP-70 (también conocida como HSP-72), HSP-90 y HSP 100/105 (Sharp et al, 1999). La HSP-70 es una chaperona que ayuda a la restauración de la estructura y la función de las proteínas desnaturalizadas.

La HSP-70 es la más inducible y se encuentra en todas la células vivas (Yenari et al, 1998). Tras el choque térmico aumenta su síntesis hasta un punto en que llega a ser la

proteína más abundante de las células. Esta proteína se sintetiza en respuesta al calor, metales pesados, toxinas, isquemia y otros tipos de estrés (Welch et al, 1996). Una vez se ha sintetizado la HSP-70 se une a proteínas desnaturalizadas (Schumacher et al, 1996) e intenta restaurar su estructura terciaria o su actividad enzimática. En el núcleo isquémico la HSP-70 se produce principalmente en las células endoteliales, las células que son más resistentes a la isquemia, mientras que en la zona de penumbra la HSP-70 se expresa en neuronas. La expresión de HSP-70 fuera de la zona del infarto puede ser utilizada para definir una de las varias penumbras isquémicas (Hossmann, 1994), indicando con esta expresión la zona de desnaturalización proteica en las áreas de isquemia. La producción aumentada de HSP-70 in vivo protege al cerebro del daño producido por la isquemia y las crisis prolongadas (Yenari et al, 1998).

La HSP-32, también conocida como hemooxigenasa-1 (HO-1), se sintetiza principalmente por la microglía y es una de las varias proteínas que metabolizan el grupo hemo a monóxido de carbono, hierro y biliverdina. La HO-1 regula el recambio de la proteína hemo, el metabolismo del hierro y el estrés oxidativo, suprime el estrés oxidativo a través de la acción de la bilirrubina, y posibilita la modulación del monóxido de carbono en el endotelio capilar. La HO-1 se sintetiza en respuesta al choque térmico, el hemo y el estrés oxidativo (Massa et al, 1996). La microglía recoge las proteínas hemo extracelulares tras la lisis celular o la hemorragia. Una vez en la microglía, el grupo hemo induce la transcripción de HO-1 que, entonces, metaboliza el hemo a biliverdina, monóxido de carbono y hierro. La liberación de hierro por la HO-1 se unirá a la ferritina, quizá mediante una función chaperona de la HO-1 (Poss et al, 1997). La producción en exceso de HO-1 protege a los vasos contra el daño mediado por el hemo y la hemoglobina (Abraham et al, 1995). Así, la HO-1 podría también proteger al cerebro contra el daño mediado por la sangre y la hemoglobina.

Propiedades antiinflamatorias de la heparina no fraccionada.

La inhibición de la inflamación ha sido propuesta como una de la contribuciones beneficiosas de la HNF administrada a pacientes con ictus isquémico agudo (Chamorro, 2001). Otros efectos biológicos adicionales, como la regulación de la angiogénesis, la modulación de la lipoproteín lipasa, el mantenimiento de la competencia del endotelio y la inhibición de la proliferación del músculo liso vascular tras la lesión endotelial, podrían

también tener implicaciones clínicas (Arfors et al, 1993; Clowes et al, 1977; Diamond et al, 1995; Sy et al, 1983).

Existe una evidencia creciente, tanto clínica como experimental, que indica que la HNF posee propiedades antiinflamatorias (Tyrrell et al, 1999). La heparina tiene un papel regulador en la inflamación, limitando la activación celular y el subsiguiente daño celular y remodelado (Page, 1991). Los efectos antiinflamatorios incluyen la unión a Mac-1, la inhibición del rodamiento leucocitario, el bloqueo de las selectinas y la atenuación de la iNOS y de la liberación del óxido nítrico (Kitamura et al, 1996; Nelson et al, 1993; Bazzoni et al, 1992). La HNF también modula la adhesión de células mononucleares a las células vasculares in vitro (Smailbegovic et al, 2001). Estudios en cultivos celulares indican que la HNF interfiere con la mediación normal de la adhesión celular, impidiendo la adhesión de la P-selectina a neutrófilos (Skinner et al, 1991) y atenuando la adhesión de neutrófilos y células mononucleares a las células endoteliales vasculares humanas activadas por endotoxina (Kitamura et al, 1996). Además, la habilidad de la HNF de inhibir la expresión de la heparanasa de los linfocitos T se correlaciona con una disminución de la hipersensibilidad retardada en ratones (Lider et al, 1990).

La HNF se une a varias integrinas y moléculas de adhesión involucradas en la entrada de células periféricas en los tejidos, incluyendo la Mac-1 (CD11b/CD18; Diamond et al., 1995), la L-selectina (Koenig et al., 1998), la P-selectina (Wang et al., 2002), y la molécula de adhesión plaquetaria endotelial-1 (Watt et al., 1993).

La HNF modula la adhesión de las células mononucleares in vitro e inhibe la generación de especies reactivas de oxígeno in vivo por los leucocitos polimorfonucleares y los monocitos (Dandona et al., 1999). Esto podría ser relevante en el contexto de la isquemia-reperfusión cerebral, en la que monocitos y macrófagos tienen el potencial de iniciar y amplificar los eventos trombogénicos mediante la expresión de factor tisular en su superficie celular (Edwards et al, 1979). Además, la HNF atenúa la inducción de moléculas de adhesión vasculares, el factor de von Willebrand y la trombospondina en las células endoteliales mediante la IL-1 (Minter et al, 1996) y suprime la producción monocitaria de varias citocinas inducida por el lipopolisacárido (Hogasen and Abrahamsen, 1995). La HNF elimina el aumento en monocitos marcados positivamente con factor tisular tras la inducción in vivo por endotoxina y aumenta los niveles plasmáticos del inhibidor de la vía del factor tisular (Pernestorfer et al, 1999). La HNF

disminuye los niveles plasmáticos elevados de factor tisular y la actividad procoagulante monocitaria en la angina inestable (Gori et al, 1999).

Tras la liberación de citocinas, el factor tisular es el iniciador celular primario de la cascada de la coagulación in vivo y representa una envoltura hemostática que se expresa difusamente en el córtex y los vasos cerebrales (Drake et al, 1989). De esta forma, cuando se produce el daño cerebral isquémico, la exposición del factor tisular a la sangre circulante provoca un estado protrombótico transitorio. Los astrocitos y las células endoteliales del tejido infartado expresan intensamente el factor de adhesión VCAM-1 (Blann et al, 1999), que induce la expresión de factor tisular (FT) (McGilvray et al, 1997). Dada la expresión rica y diseminada del FT en el córtex cerebral y vasos intracraneales, cualquier daño isquémico agudo podría iniciar la cascada de la coagulación, independientemente del tipo de ictus (Chamorro, 2001). Teóricamente, la HNF podría ser útil en la mayoría de pacientes si el fármaco previniera los mecanismos proinflamatorios descritos previamente.

Tras interactuar con la superficie celular, la HNF protege el endotelio del daño ocasionado por los radicales libres (Hiebert et al, 1990; Hiebert et al, 1991). La HNF y otros polianiones relacionados, como el dermatán y el heparán sulfato, también son capaces de liberar diamina oxidasa y superóxido dismutasa desde la superficie endotelial (Robinson-White et al, 1985; Karlsson et al, 1987). Por lo tanto, la HNF podría tener también propiedades antioxidantes.

La inhibición de la inflamación podría ser parte de los efectos beneficiosos de la HNF en la cardiopatía isquémica (Ott et al, 1996) y en varios modelos preclínicos de enfermedades inflamatorias como el asma, enfisema, síndrome de distrés respiratorio del adulto (ARDS), hipersensibilidad retardada, encefalitis autoinmune experimental, daños por isquemia-reperfusión y shock circulatorio (Tyrrell, et al, 1999). Existe evidencia de que el tratamiento con HNF es beneficioso en el asma, ARDS, colitis ulcerosa y artritis reumatoide. Además, la heparina reduce las metástasis del células carcinomatosas en modelos animales y en humanos con enfermedad neoplásica (Zacharski et al, 1998).

En modelos de isquemia cerebral también se ha evidenciado un efecto neuroprotector de la HNF. Así, en un modelo de isquemia cerebral en monos, con oclusiones arteriales de 15 y 30 minutos, se demostró que dosis altas de HNF (5 mg/kg, 1-2 minutos antes de la

oclusión) reducían el volumen de infarto (Cromwell et al, 1955; Cromwell et al, 1958). La HNF las HBPM y el heparán sulfato han sido evaluados en un modelo de isquemia focal transitoria en roedores, en la que los animales fueron sometidos a 1 hora de isquemia y 48 horas de reperfusión (Yanaka et al, 1996). Los grupos de tratamiento que recibieron HNF y dextrán sulfato mostraron una reducción significativa en la acumulación de neutrófilos, tamaño del infarto y disfunción neurológica a las 48 horas del a reperfusión. La protección neuronal fue dependiente de las propiedades antileucocitarias más que de la actividad anticoagulante de los compuestos. El tratamiento con 4 mg/kg de HNF administrada a los tiempos 0 y 24 horas demostró la mayor protección en términos de reducción de infiltración de neutrófilos, del tamaño del infarto y de la afectación neurológica. La HNF también demostró menor efecto protector administrada a las 3 y 24 horas y ningún beneficio cuando se administraba a las 6 y 24 horas. La HNF fue más eficaz que las dosis equivalentes de HBPM (Yanaka et al, 1996). Por otro lado, en pacientes con ictus isquémico agudo los niveles plasmáticos de citocinas proinflamatorias son mas bajos en los que reciben tratamiento anticoagulante que en los antiagreagados (Vila et al, 2000).

II. OBJETIVOS

1. Establecer un modelo animal de tratamiento continuo con heparina no fraccionada que permita mantener una heparinemia dentro de los márgenes terapéuticos deseados. Además, determinar si mediante el test del tiempo de tromboplastina parcial activada podemos predecir el nivel de heparina.

2. Determinar si la heparina no fraccionada administrada de la manera adecuada es neuroprotectora en un modelo de isquemia-reperfusión cerebral focal en rata.

3. Estudiar los mecanismos inflamatorios que facilitan el daño por reperfusión en la isquemia cerebral focal para evaluar el posible efecto antiinflamatorio de la heparina no fraccionada y explicar el eventual efecto neuroprotector.

4. Evaluar marcadores séricos de inflamación en pacientes con ictus isquémicos agudos para determinar si existe un efecto antiinflamatorio al usar heparina no fraccionada en contraposición con otros tratamientos. Además, estudiar el pronóstico funcional de estos pacientes para dilucidar si este potencial efecto antiinflamatorio tiene una repercusión clínica.

5. Elaborar un ensayo clínico aleatorizado comparando la heparina no fraccionada con la aspirina en el tratamiento del ictus isquémico agudo para evaluar mediante las medicina basada en la evidencia la posible superioridad de la heparina no fraccionada debido a su potencial efecto neuroprotector mediado por mecanismos antiinflamatorios.

III. TRABAJOS

1. Steady plasma concentrations of unfractionated heparin reduces infarct volume and prevents inflammatory damage after transient focal cerebral ischemia in the rat.

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Steady Plasma Concentration of Unfractionated Heparin Reduces Infarct Volume and Prevents Inflammatory Damage After Transient Focal Cerebral Ischemia in the Rat

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Unfractionated heparin (UH) decreases the extent of infarction after transient focal brain ischemia in the rat and abridges neuroinflammatory damage in patients with acute stroke. This study was aimed at assessing whether controlled and steady heparinemia in plasma can reduce infarct volume and exert neuroprotective effects after ischemia. Infarct volume was measured at 24 and 7 days following a 1-hr intraluminal middle cerebral artery (MCA) occlusion in rats treated with UH or with vehicle. After testing several UH administration protocols, we choose to give a bolus of 200 U/kg, which was started 3 hr after the occlusion, followed by a 24-hr intraperitoneal perfusion of 70 U/kg/hr, which maintained a 24-hr steady plasma heparinemia (0.3–0.6 U/ml) and caused no CNS or systemic bleeding. In addition, plasma IL-10 concentration was measured by ELISA, endothelial VCAM-1 expression was evaluated by i.v. injection of a ^{125}I-labeled monoclonal antibody against VCAM-1, and brain hemeoxygenase-1 (HO-1) expression was determined by Western blot. UH-treated rats showed smaller infarctions than rats treated with vehicle, as well as higher IL-10 plasma levels and HO-1 brain expression and lower endothelial VCAM-1 induction. The study shows that a stable plasma concentration of UH given at nonhemorrhagic doses reduces infarct volume after ischemia-reperfusion in the rat. It also shows that UH prevented the induction of cell adhesion molecules in the cerebral vasculature and increased the expression of molecules with antiinflammatory and prosurvival properties. These findings support further testing of the clinical value of parenteral, adjusted, high-dose UH in patients with acute stroke.

Key words: inflammation; ischemia-reperfusion; VCAM-1; IL-10; HO-1

On theoretical grounds, unfractionated heparin (UH) is given to patients with acute ischemic stroke to improve neurological outcome, prevent thrombus progression, facilitate collateral circulation, hinder the development of early stroke recurrence, and prevent deep venous thrombosis or pulmonary embolism (Sherman et al., 1995). Although some experts have warned against the use of heparin in acute stroke (Gubitz et al., 2000; Coull et al., 2002), the available information is confined to the value of unadjusted doses (from low to moderate) of subcutaneous UH (International Stroke Trial Collaborative Group, 1997). Contrarily, the value of adjusted high-dose i.v. UH, which represents the most usual mode of heparin administration, has never been evaluated in a randomized clinical trial. Moreover, although it is acknowledged that the treatment of acute stroke has to be implemented as diligently as possible, this urgency was disregarded without exception in the trials that assessed the value of UH (Chamorro et al., 1999; Chamorro, 2001). Despite the lack of adequate evidence of the clinical value of UH, a considerable number of physicians use worldwide UH in acute stroke (Al-Sadat et al., 2002), showing the need for clinical trials addressed to assess the clinical value of ad-

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justed high-dose i.v. UH in acute stroke (Chamorro, 1999). In this regard, there is an ongoing academic study, the Rapid Anticoagulation Prevents Ischemic Damage (RAPID; see Major Ongoing Stroke Trials, 2003), which will eventually contribute to the unraveling of this issue.

Only limited numbers of experimental studies have addressed the question of whether UH has potential benefits in animal models of cerebral ischemia. In a murine model of transient focal ischemia, acute administration of UH reduced the extent of brain infarction (Yanaka et al., 1996a,b). As expected, higher anticoagulant doses (Yanaka et al., 1996a) and shorter anticoagulation delays (Yanaka et al., 1996b) were the most effective. Experimental and clinical studies indicate that the effects of UH go beyond anticoagulation (Tyrrell et al., 1999; Lever and Page, 2002). Several clinical studies have also shown that the total leukocyte count (Chamorro et al., 2000) and the levels of several proinflammatory cytokines and adhesion molecules were lower in stroke patients anticoagulated with adjusted high-dose i.v. UH than in those treated with aspirin (Chamorro et al., 2002). Importantly, these molecular effects were paralleled in these studies by a greater rate of clinical recovery and a lower risk of worsening in anticoagulated individuals (Chamorro et al., 2002). These findings agree with the current view recognizing neuroinflammation as an important therapeutic target in clinical stroke (Dirnagl et al., 1999; Vila et al., 2000). We designed the present study, based on the above-mentioned data, to confirm the neuroprotective effect of UH in a model of transient focal ischemia in the rat by using doses equivalent to those used in clinical practice and to inquire whether this potential neuroprotection relies on the modulation of inflammatory mechanisms.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Iffa-Credo, Lyon, France; n = 102; 280–320 g body weight) were used. Rats were housed under a 12-hr day/night light cycle and had free access to food and water. Animal experiments were conducted with the approval of the ethical committee of our institution, in compliance with the Spanish legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes" and in accordance with the Directives of the European Union.

Drugs

Porcine UH was purchased from Rovi (Spain) as a 5% solution. All chemicals, unless otherwise stated, were from Sigma (St. Louis, MO).

Pharmacokinetic Studies

We sought to administer a dose of UH that caused a stable heparinemia (0.3–0.6 U/ml of plasma) through the 24-hr duration of the study. For this purpose, different administration protocols were tested, and heparinemia and activated partial thromboplastin time (aPTT) were measured in seriated plasma samples, as described below. Blood samples (0.9 ml) were obtained from the femoral vein and were immediately mixed with 0.1 ml citrate. Samples were then centrifuged at 9,000*g* for 3 min to obtain plasma, which was kept frozen at −80°C until further biochemical analyses. Heparinemia was measured by a spectrophotometric reaction at 450 nm that uses FXa and a chromogen as substrates (Coamatic Heparin, West Chester, OH). Results are expressed as heparin activity (U/ml plasma). The administration protocols that we studied were as follows. 1) We initially studied the effect of an i.v. bolus of 300 U/kg body weight (n = 4), and blood samples were taken at 10, 30, 60, 120, and 240 min. 2) We used osmotic pumps (Alzet Osmostic Pump 2ML1; Alza, Palo Alto, CA) with a constant flow of 10 μl/hr that were placed in the intraperitoneal (i.p.) space to infuse heparin for the 24-hr study. All rats (n = 23) received an i.v. bolus of UH (100, 200, or 300 U/kg), followed by implantation of an i.p. pump delivering UH (30, 50 or 70 U/kg/hr). Blood samples were extracted at 0.5, 2, 4, 5, 6, 7, 8, and 24 hr.

Intraluminal Middle Cerebral Artery Occlusion/ Reperfusion in the Rat

Focal brain ischemia was produced by a 1-hr intraluminal occlusion of the middle cerebral artery (MCA), with reperfusion, as reported (Justicia et al., 2001). Briefly, rats were anesthetized with halothane (4%) and intubated through the trachea for controlled ventilation. Mean arterial blood pressure was monitored, and body temperature was maintained between 36.5°C and 37.5°C during surgery. A filament (nylon monofilament 3/0; Suturas Aragó Spain) was introduced (22 mm) through the external carotid artery to the level where the MCA branches out. In addition, the ipsilateral and contralateral common carotid arteries (CCA) were clamped. After this procedure, the blood flow derived from collateral circulation was minimized. The microclip on the contralateral CCA was removed 50 min later to minimize the risk of hemorrhage at reperfusion. Ten minutes later, i.e., 1 hr following MCA occlusion, the filament was cautiously removed, and the clip of the ipsilateral CCA was taken off to allow reperfusion, as previously assessed (Soriano et al., 1997). Rats were maintained in an anesthetized state until the beginning of the treatment and were then allowed to recover for 24 hr or 7 days. Blood samples were obtained at 24 hr for measurement of the content of interleukin (IL)-10 in plasma. Rats were then euthanized, and the brain was removed from the skull and further processed either for measurements of infarct volume or for biochemical determinations.

UH Treatment in Ischemic Rats

Treatment with UH was initiated 3 hr following MCA occlusion, i.e., after 2 hr of reperfusion. According to the previous kinetic studies, an i.v. bolus of 200 U/kg was given through the femoral vein, and an infusion pump (Alzet) was placed in the i.p. cavity to deliver 70 U/kg/hr. Control rats received a similar injection of vehicle (saline). The infusion treatment was maintained until the rat was killed (either 1 day or 7 days).

Evaluation of Infarct Volume

At 24 hr (n = 26) or at 7 days (n = 14), rats were anesthetized and decapitated. The brain was extracted from the skull and serially sectioned in 2-mm coronal sections that were stained for 10 min with 2,3,5-triphenyltetrazolium chloride

(TTC) 2% at 37°C, followed by an overnight fixation with 4% paraformaldehyde. Infarcted areas were measured in each section (mm^2) with an image-analysis system (AIM; Image Research), and areas were integrated to obtain the infarct volume (mm^3). Studies were carried out in a blind fashion; the investigator carrying out infarct volume determinations was unaware of the treatment assigned to each rat.

Assessment of Cerebral Blood Flow

Cortical blood flow was determined by laser Doppler flowmetry in certain rats, as previously reported (Soriano et al., 1997; Justicia et al., 2001). On the day prior to ischemia, anesthesia was induced with 4% halothane (Fluothane; Zeneca) in a mixture of 70% N_2O and 30% O_2 through a face mask. Rats were placed in the prone position on a stereotaxic frame (Kopf Instruments), and anesthesia was maintained with 1.5–2% halothane. After a cranial midline skin incision, a stainless steel cannulae was implanted in the right lateral ventricle through a burr hole at the following coordinates (Paxinos and Watson, 1986): 0.3 mm posterior, 1.2 mm lateral, 3.2 mm ventral to bregma. A truncated 21-G needle was implanted in a burr hole to measure cortical perfusion. The needle was placed at the following coordinates: 2.0 mm posterior, 3.5 mm right to bregma. Both the cannulae and the truncated needle were fixed to the skull with two miniature screws and dental cement. The wound was sutured and the animal allowed to recover for 24 hr. On the following day, the flexible plastic tip (0.5 mm in diameter; PF319; Perimed) of a master laser-Doppler probe (PF418; Perimed) was introduced through the cranial 21-G needle until the tip of the probe touched the brain surface, thus measuring the frontoparietal cortical perfusion (CP) in the territory of the MCA by means of a laser-Doppler flowmeter (PF4001 Master; Perimed). Basal flow was recorded, and then focal cerebral ischemia was produced by transient intraluminal occlusion of the MCA, as described above. Measurement of blood flow was continuously carried out during the 1 hr of MCA occlusion, during the following 2 hr of reperfusion prior to treatment, and during the third hour corresponding to the first hour of treatment with either UH or vehicle. Body temperature was monitored with a rectal probe for up to 3–4 hr after MCA occlusion.

Plasma Levels of IL-10

IL-10 content (pg/ml) was evaluated in plasma with an ELISA (Rat IL-10 ELISA Kit; Pierce Endogen, Rockford, IL), following the instructions of the manufacturers. Plasma was obtained 24 hr after MCA occlusion/reperfusion from the groups of rats that were used to study infarct volume.

Endothelial Expression of VCAM-1

VCAM-1 was studied in an additional group of rats (n = 20) by a double-labeling isotopic technique described by Sans et al. (1999) that was adapted to our model of focal cerebral ischemia-reperfusion. A murine mAb Ig G2a against rat VCAM-1 (5F10; Biogen Inc., Cambridge, MA) was labeled with ^{125}I (Amersham Iberica, Madrid, Spain), and an isotype-matched mAb (UPC-10), which does not bind to rat VCAM-1, was labeled with ^{131}I (Amersham Iberica) by using the iodogen method (Sans et al., 1999). Controls (n = 6), and UH-treated (n = 7) and vehicle-treated (n = 7) rats subjected to MCA occlusion were anesthetized (ketamine/xylazine) at 24 hr, and then had the femoral artery and vein cannulated. A mixture containing 20 μg of ^{125}I-5F10 and 5 μg of ^{131}I-UPC-10 was injected through the vein. This dose of anti-VCAM-1 mAb has been shown to be saturating in previous assays (Sans et al., 1999). Five minutes later, the rat was exsanguinated, the brain was removed, the ipsilateral and contralateral MCA territories (including cortex and striatum) were dissected out, and radioactivity was measured in a gamma counter. Specific binding of mAb (ng/g of tissue) was calcutated by correcting for nonspecific binding, as reported elsewhere (Sans et al., 1999).

Expression of Hemeoxygenase-1

Another group of rats (n = 15) was subjected to ischemia and received either UH or vehicle, as described above. At 24 hr, rats were euthanized; the brain was removed; and the cortex was dissected out, rapidly frozen, and kept at −80°C. Tissue samples were homogenized in RIPA buffer containing 0.01 M phosphate-buffered saline (PBS), sodium dodecyl sulfate, sodium deoxycholate, the nonionic detergent Igepal, and a cocktail of protease inhibitors (Complete; Boehringer Mannheim, Mannhgeim, Germany). All products and reagents, unless otherwise stated, were from Sigma. Samples were kept on ice for 30 min and then centrifuged at 12,000*g* at 4°C for 15 min, and the supernatants were used as the total protein fraction. The protein concentration was determined with the Bradford assay (Bio-Rad, Hercules, CA). Fifty micrograms of the protein extracts were denatured at 100°C for 5 min and then loaded in a 10% polyacrylamide gel. Proteins were then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA), which was incubated overnight at 4°C with a mouse monoclonal antibody directed against hemeoxygenase-1 (HO-1; Stressgen, Collegeville, PA) diluted 1:4,000. On the following day, membranes were incubated for 1 hr with an anti-mouse Ig peroxidase-linked secondary antibody (1:2,000; Amersham, Arlington Heights, IL). The reaction was visualized with a chemiluminiscence detection system based on the luminol reaction. The intensity of the bands was determined by densitometric analysis (Kodak DC-120 camera and Kds1D; Digital Science System software). Values of band intensity after UH treatment are expressed as percentage of the mean value of the vehicle-treated group.

Statistical Analysis

For statistical analyses, parametric and nonparametric tests were used as appropriate. The level of significance was established at $P < .05$. Values are expressed as mean ± SEM.

RESULTS

Dose Finding for UH

A single i.v. bolus of UH at a dose of 300 U/kg resulted in plasma levels that had faded completely after 3 hr of administration. In an additional groups of rats, a bolus of 300, 200, or 100 U/kg was followed by a 24-hr i.p. perfusion of 30, 50, or 70 U/kg/hr. The best treatment regime, as shown in Figure 1, was an initial bolus of 200 U/kg, followed by i.p. perfusion of 70 U/kg/hr. Therefore, this was the UH dosing regime applied to rats

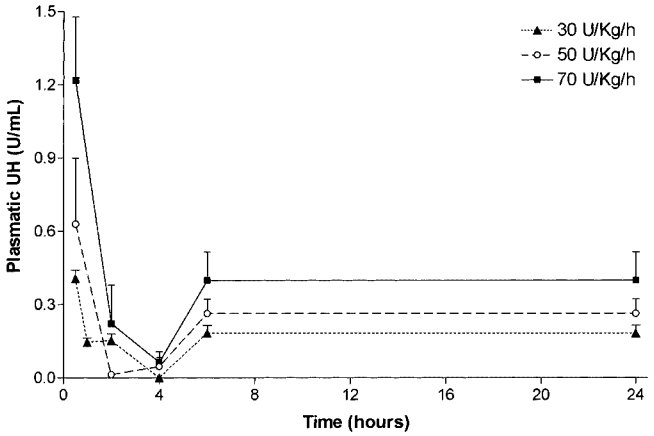

Fig. 1. Time course of plasma heparin (U/ml) after an i.v. bolus injection of UH (200 U/kg) followed by a 24-hr i.p. infusion (200 U/kg/hr; n = 9). Heparinemia is maintained stably from about 5 to 24 hr at levels ranging from 0.375 ± 0.035 at 8 hr to 0.380 ± 0.045 at 24 hr.

subjected to ischemia. In the ischemic rats (n = 13), mean heparin levels during the 24-hr treatment were 0.46 ± 0.19 U/ml, and none of the animals experienced bleeding complications. Contrarily, an initial dose of 300 U/kg resulted in i.p. bleeding during the first 24 hr of treatment. Also, an initial dose of 100 U/kg resulted in more prolonged infratherapeutic plasma levels than the selected dose.

Infarct Volume in UH-Treated Animals and Controls

A group of 13 rats was treated with UH at the dose previously described, and 13 rats received saline serum as the vehicle. Treatment was initiated 3 hr after the onset of MCA occlusion (2 hr after reperfusion). The aPTT values at 24 hr postischemia were 18.60 ± 2.68 sec in vehicle-treated animals and 37.22 ± 8.45 sec in UH-treated animals. During the treatment course, mean arterial pressure and body temperature were maintained at similar values in both treatment groups (data not shown). Central or systemic bleeding was not observed. The volumes of infarction in the UH-treated and vehicle-treated groups were 146.5 ± 35.66 mm^3 and 270.0 ± 35.86 mm^3 (*t*-test, $P < .03$), respectively. As shown in Figure 2A, UH-treated animals showed an overall volume reduction 46% greater than that in vehicle-treated rats; these figures were 51.8% in the cortex (*t*-test, $P = .03$) and 25.8% in the striatum (*t*-test, $P = .02$). We designed another experiment to test whether the protective effect of UH persisted with time. Here, UH (n = 7) or saline (n = 7) was administered at 3 hr after the beginning of MCA occlusion (as in the previous group), and rats were sacrificed 7 days later to study infarct volume. UH was given as an i.v. bolus of 200 U/kg through the femoral vein, and an infusion pump (Alzet) was placed in the i.p. cavity to deliver 70 U/kg/hr, as described above. The i.p. infusion was

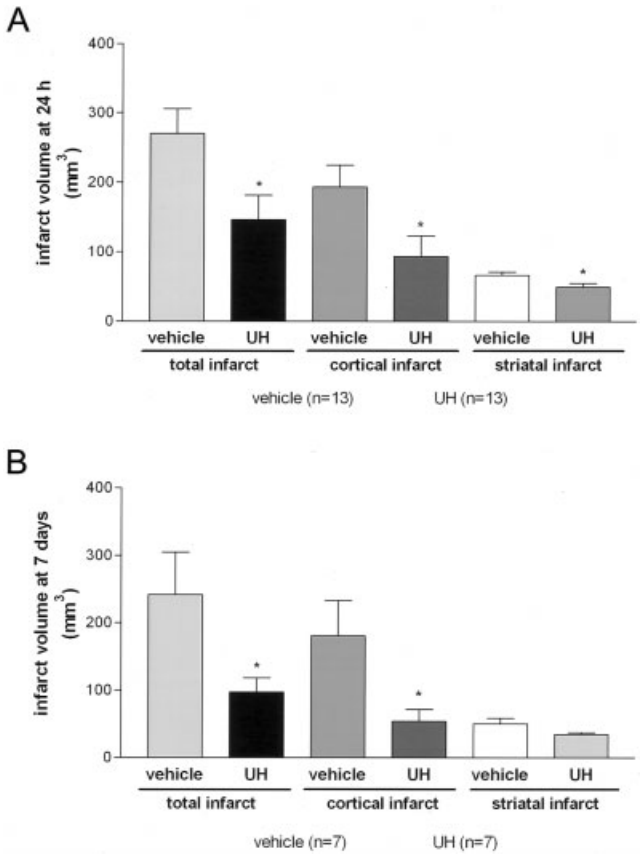

Fig. 2. Infarct volume after MCA occlusion-reperfusion in rats treated with UH or vehicle. Infarct volume was evaluated at either 24 hr (**A**) or 7 days (**B**) after MCA occlusion. Treatment was initiated at 3 hr after the beginning of the occlusion. UH significantly reduces global infarct volume in relation to the treatment controls. *UH vs. vehicle, $P < .05$.

maintained for 7 days until the rats were killed for evaluation of infarct volume. No systemic or intracerebral bleeding was observed during the 7-day treatment. Here, again, UH significantly reduced (*t*-test, $P < .05$) mean ± SEM infarct volume from 242.1 ± 62.72 mm^3 in the controls to 97.3 ± 20.95 mm^3 (Fig. 2B). The protective effect of UH was more marked in cortical than in subcortical regions (two-way ANOVA, treatment-by-region interaction factor, $P < .05$; see Fig. 2B).

Mean ± SD body temperature was monitored during ischemia and during the first 2 hr of reperfusion (i.e., before treatment was initiated); it was 37.1°C ± 0.46°C in controls and 37.0°C ± 0.46°C in the group that would later receive UH (at 3 hr after MCA occlusion). During the first 1 hr of treatment, body temperature was 37.0°C ± 0.32°C in controls and 36.8°C ± 0.22°C in the rats receiving UH. No differences were found between groups.

Heparin Does Not Alter Postischemic Blood Flow

Cortical CBF values (% of basal, mean ± SD), as measured with laser Doppler, for the vehicle-treated rats

were 30.5 ± 15.3, 134.4 ± 41.6, and 128.7 ± 22.1 during MCA occlusion, during the first 2 hr after reperfusion (before treatment was initiated), and during the third hour of reperfusion (the first hour of treatment), respectively. The corresponding values for UH-treated rats were 35.3 ± 8.1, 126.6 ± 19.5, and 133.3 ± 35.6. No differences became apparent between groups during the first 3 hr of reperfusion, indicating that the protective effect of UH was not due to postischemic blood flow enhancement.

Inflammatory Markers in the UH- and Vehicle-Administered Groups

As depicted in Figure 3A, plasma levels of IL-10 were significantly higher in the UH group (*t*-test, $P = .005$). Figure 3B also shows the expression of VCAM-1 as assessed by the binding of an mAb to the tissue (ng mAb/g) in nonischemic controls and in the ipsilateral hemisphere of ischemic rats treated with either vehicle or UH. The two groups of ischemic rats showed significantly higher expression of VCAM-1 in the ipsilateral hemisphere than nonischemic controls (ANOVA, $P < .001$). However, the expression of VCAM-1 in the ipsilateral hemisphere of animals receiving UH was reduced by 27.4% (post hoc Bonferroni's multiple-comparison test, $P < .05$) compared with the expression observed in vehicle-treated rats. Ischemia-induced brain expression of HO-1 was significantly higher in the ipsilateral hemisphere of rats receiving UH than in those receiving the vehicle (*t*-test, $P = .04$) as shown in Figure 4A,B.

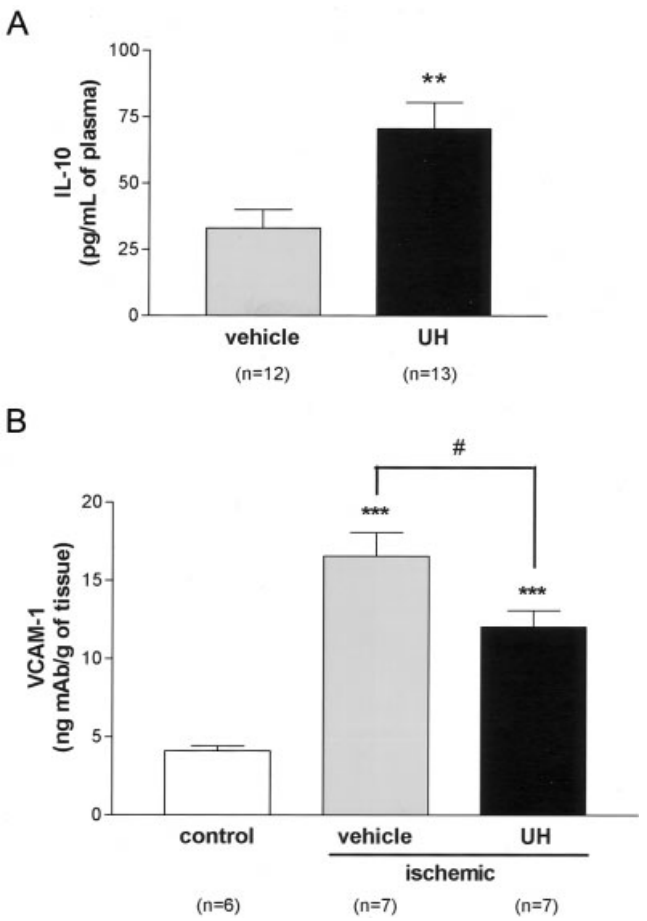

Fig. 3. **A:** Plasma IL-10 concentration (pg/ml) at 24 hr after MCA occlusion-reperfusion in rats treated with UH or vehicle. The group of rats used to determine IL-10 was the same as for Figure 2. IL-10 is significantly (**UH vs. vehicle, $P < .01$) increased in the rats that received UH. **B:** Expression of VCAM-1 at 24 hr in control rats and in rats subjected to ischemia-reperfusion that were treated with UH or vehicle. Treatment was initiated at 3 hr after induction of ischemia. VCAM-1 was evaluated in a specific group of rats systemically injected radiolabeled mAb at 24 hr. This is a double-radiolabeling technique in which a saturating dose of ^{125}I-labeled mAb against VCAM-1 is injected together with a ^{131}I-labeled nonspecific mAb, which is used to correct for nonspecific binding (see Materials and Methods). VCAM-1 expression is assessed though the specific binding of the mAb. Values are expressed as ng mAb/g tissue. Ischemia highly induces the expression of VCAM-1 (***ischemic vs. control, $P < .001$), and this effect is significantly ($^{\#}$UH vs. vehicle, $P < .05$) attenuated in the rats that received UH.

DISCUSSION

In this study, we found that a steady plasma heparinemia achieved by UH treatment initiated at 3 hr after the onset of ischemia reduces infarct volume. Moreover, the results show that neuroprotection is associated with a favorable modulation of several inflammatory processes, including a lower induction of VCAM-1 and increased plasma concentration of IL-10, together with an enhanced expression of an antiinflammatory and neuroprotective heat shock protein. In agreement with previous studies (Yanaka et al., 1996a,b), we found that rats receiving UH after 1 hr of MCA occlusion and 2 hr of reperfusion developed smaller brain infarctions than animals treated with vehicle, both in cortical areas and in the striatum. The importance of the dose of UH and its mode of administration were emphasized at the inception of the present study and corroborated later in the results. Indeed, we observed that dose regimes other than that corresponding in the rat to the dose targeted in clinical practice resulted in insufficient plasma levels of the agent or produced an unacceptable risk of bleeding complications. The selected dose of UH achieved rapid plasma levels and stable values during all the reperfusion period. To our knowledge, our study is the first to substantiate in the rat model of brain focal ischemia the dose of UH most frequently used in clinical practice. Our findings concur with previous studies stressing the safety of close monitoring of UH in the clinical setting (Chamorro et al., 1999), a practice that has been disregarded in randomized anticoagulation trials.

The relevance of inflammatory mechanisms in acute ischemic stroke has been extensively proved in preclinical (del Zoppo et al., 1991; Ley et al., 1991; Kochanek and Hallenback, 1992; Bazzoni et al., 1993; Silvestro et al., 1994; Ryu et al., 1996; Xie et al., 1997; Lever et al., 2000) and clinical (Chamorro et al., 2000, 2002; Vila et al., 2000) studies. We now provide new information suggesting that

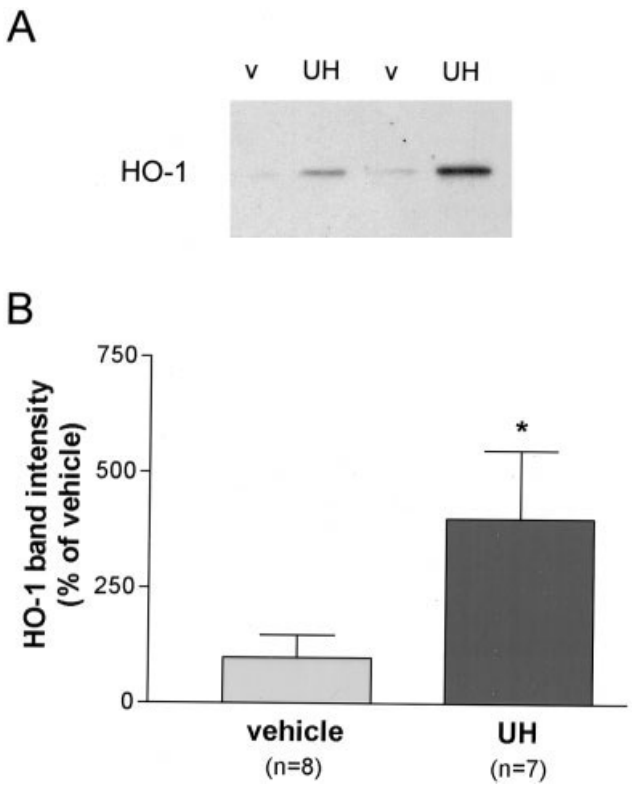

Fig. 4. **A:** Expression of HO-1 at 24 hr following ischemia/reperfusion as determined by Western blot. HO-1 is barely detected in the control brain (not shown), but low expression is detected as a faint 32-kDa band in the ipsilateral hemisphere of ischemic rats that received the vehicle (v). Treatment with UH greatly increases the intensity of the HO-1 band in the ipsilateral hemisphere after ischemia. Two representative animals are shown for the vehicle (lanes 1 and 3) and UH (lanes 2 and 4) groups. **B:** Measurement of band intensity reveals a significant (*UH vs. vehicle, $P < .05$) increase of HO-1 in the UH-treated ischemic group in relation to the vehicle-treated ischemic group.

UH is in fact a neuroprotective agent with antiinflammatory properties in acute focal ischemia, which might be beneficial in practice if the agent is given as described. UH is known to bind to a number of integrins and adhesion molecules involved in peripheral cell trafficking into tissues, including Mac-1 (CD11b/CD18; Diamond et al., 1995), L-selectin (Koenig et al., 1998), P-selectin (Wang et al., 2002), and platelet endothelial adhesion molecule-1 (Watt et al., 1993). UH modulates the adhesion of mononuclear cells to vascular endothelial cells in vitro, in agreement with these findings, and inhibits reactive oxygen species generation by both polymorphonuclear leukocytes and monocytes in vivo (Dandona et al., 1999). This might be relevant in the context of brain ischemia-reperfusion, in that monocytes and macrophages have the potential to initiate and amplify thrombogenic events by the expression of tissue factor on their cell surface (Edwards et al., 1979). Furthermore, UH attenuates the induction of vascular adhesion molecules, von Willebrand factor, and thrombospondin in endothelial cells by IL-1 (Minter et al., 1996) and suppresses lipopolysaccharide-induced monocyte production of several cytokines (Hogasen and Abrahamsen, 1995). Also, in rat microvascular brain endothelial cells, UH reduces the cytokine-induced high-output nitric oxide (NO) synthesis by attenuation of inducible NO synthase (iNOS) activity, whereas basal constitutive NO generation is not influenced (Bonmann et al., 1998).

A new finding of this study was that the administration of UH was associated with increased plasma levels of IL-10, an antiinflammatory molecule mainly secreted by lymphocytes and monocytes/macrophages that can suppress the production of proinflammatory molecules, such as tumor necrosis factor (TNF)-α, IL-1β, and IL-8 (Tedgui and Mallat, 2001). Administration of IL-10, either systemically into a vein or centrally into the lateral ventricle (Spera et al., 1998), protects tissue exposed to a variety of ischemia-reperfusion regimes by blocking gene transcription and down-regulating the release of intercellular adhesion molecule-1 (ICAM-1) and matrix metalloproteinases. Recently, lower levels of IL-10 in plasma were measured in patients with early deteriorating ischemic stroke (Vila et al., 2003).

The beneficial effects of UH also included a lower expression of endothelial VCAM-1. Intense expression of VCAM-1 by astrocytes and endothelial cells has been observed from the infarcted tissue, but not from noninfarcted areas (Blann et al., 1999). Although there are marked longitudinal changes in soluble cell adhesion molecule concentrations in patients with acute ischemic stroke (Bitsch et al., 1998), a recent clinical study showed a lower increase of VCAM-1 in patients treated with UH early after symptom onset (Chamorro et al., 2002). VCAM-1 is an adhesion molecule that is expressed following endothelial activation by cytokines and selectively binds mononuclear cells such as lymphocytes and monocytes. Contrarily, resting endothelial cells express minimal or undetectable levels of VCAM-1. VCAM-1 could facilitate brain damage through the expression of tissue factor, insofar as ligation of monocyte integrins to endothelial VCAM-1 induces tissue factor expression (McGilvray et al., 1997). UH abrogates the endotoxin-induced increase in tissue factor-positive monocytes in vivo (Pernerstorfer et al., 1999).

The administration of UH was also associated with increased cerebral postischemic expression of the inducible intracellular molecular chaperone HO-1, which is involved in cell survival and recovery after injury (Sharp et al., 1999). HO-1 regulates heme protein turnover, iron metabolism, and oxidative stress. Overproduction of HO-1 protects the vessels against heme- and hemoglobin-mediated injury (Mazza et al., 2003), hydrogen peroxide-mediated astrocytes cell death (Fauconneau et al., 2002), and focal cerebral ischemia (Panahian et al., 1999). Also, intramyocardial injection of the HO-1 gene in normal rat heart prior to acute coronary artery ligation-release causes an important reduction in left ventricular myocardial infarction (Melo et al., 2002). HO-1 expression is increased

in cultured endothelial cells by aspirin treatment, suggesting that induction of HO-1 may be a novel mechanism by which aspirin prevents cellular injury under inflammatory conditions (Grosser et al., 2003). In agreement with this view, our results showing an increased expression of HO-1 in animals treated with UH can be taken as a surrogate for increased cell survival in the rat brain after treatment with UH.

The structural features of heparin that are involved in the beneficial actions of this compound against brain ischemia remain unknown. The use of heparan sulfate analogue libraries that are produced chemicoenzymatically from heparin (Yates et al., 2004) might help to unravel the essential parts of this molecule responsible for its biological actions.

In summary, this study provides strong evidence corroborating the capacity of parenteral UH to reduce the extent of brain infarction in the rat exposed to focal ischemia/reperfusion. Beyond this finding, the present results show that UH increases neural cell survival, induces a stronger antiinflammatory response, and decreases the interaction of circulating cells with the inflammed endothelium. Further experiments should be undertaken to unravel the molecular mechanisms underlying UH-induced brain protection, the action of UH on the recovery of brain function, and the neuroanatomical correlates of the antiinflammatory effects of this molecule. Adequate dosing of UH for an effective and safe administration is also emphasized in the present study. Taken altogether, these results are in concurrence with recent observational clinical series that ascribe salutary effects to weight-adjusted UH in patients with acute ischemic stroke. Nonetheless, these results should not be extrapolated to the bedside before randomized studies of adjusted-dose i.v. administration of UH in acute stroke are completed.

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Comparison of the acute-phase response in patients with ischemic stroke treated with high-dose heparin or aspirin

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Abstract

Experimental studies have suggested that unfractionated heparin (UH) has antiinflammatory properties. It is unknown whether UH also has these properties in patients with acute ischemic stroke. Within 12–24 h of treatment onset we measured the acute-phase response as reflected by the erythrocyte sedimentation rate (ESR) and total number of leukocytes ($\times 10^9$/l) in 706 consecutive patients with acute ischemic stroke treated with full-dose UH ($n=450$), or 300 mg/day aspirin ($n=256$). Clinical outcome (Mathew scale) at hospital discharge and the effect of factors such as treatment (UH and aspirin), and acute phase response were assessed using multivariate analyses adjusted for baseline confounders and incident complications. Separate models were created for patients with lacunar and nonlacunar stroke. Whereas there were not differences at baseline between the two treatment groups, total leukocyte counts (8.0 ± 4.1 vs. 8.6 ± 3.2, $P<0.01$) and ESR (21.7 ± 20.9 vs. 25.2 ± 22.9, $P<0.05$) were statistically significantly lower in patients treated with UH. This effect of UH was more accentuated in patients with nonlacunar stroke. Overall, leukocytes (7.2 ± 2.3 vs. 8.4 ± 4.0, $P<0.01$), and ESR (15.7 ± 17.2 vs. 24.3 ± 22.2, $P=0.0001$) were lower in patients with complete early recovery and this effect was restricted to patients with nonlacunar stroke. Whereas baseline impairment, symptomatic bleeding and stroke recurrence were independent negative outcome predictors, the use of UH was positively associated with early recovery in all patients. This study shows that full-dose UH reduces the acute-phase reaction that follows ischemic stroke more effectively than aspirin. The prognostic implications of such effect seem more notable in patients with nonlacunar stroke.

Keywords: Stroke treatment; Heparin; Aspirin; Leukocytes

1. Introduction

During the second half of the 1990s four trials of anticoagulation after acute ischemic stroke were reported in final [1–3] or preliminary form [4]. With one single exception [1], these studies showed that low- or medium-dose unfractionated heparin (UH) [2], nadroparin calcium [4], and danaparoid sodium [3] provided no clear clinical benefits to the patients, or resulted in an excessive risk of untoward complications. However, the interpretation of these results are limited by the design of the trials, which allowed treatment allocation before the performance of a brain CT scan [2], evaluated patients with varied stroke subtypes including a large proportion of lacunar strokes [1–4], and lacked monitoring of the level of anticoagulation achieved in treated patients [1,2,4]. Maybe because of these limitations many stroke experts still use UH at their institution to prevent thrombus progression, and hinder the occurrence of early stroke recurrence, deep venous thrombosis and pulmonary embolism [5,6].

The antithrombotic effects of UH are mediated largely through its interaction with antithrombin III that accelerates its ability to inactivate factor IIa, factor Xa, and factor IXa, respectively [7]. Recent data collected in patients with inflammatory conditions, such as Crohn's disease or ulcerative colitis, also suggest that UH possesses antiin-

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flammatory properties [8–10]. These properties have also been convincingly demonstrated in murine models of brain ischemia [11], which also showed that UH was neuroprotective in a dose- and time-dependent manner [12]. Hence, the modulation of inflammatory markers was only, achieved if UH was administered to the animal at full doses and during the first hour following the onset of brain ischemia [11,12]. To our knowledge, whether UH also modulates the inflammatory response that occurs in patients with acute ischemic stroke is unknown. Prompted by these encouraging observations we assessed the relative capacity of UH and aspirin to modulate the acute-phase response that follows ischemic stroke. As the current view of the pathophysiology at brain ischemia emphasizes the deleterious role of inflammation, especially during reperfusion [13], the present observational study is justified. Specific questions addressed by this study are: do aspirin and UH modulate to the same degree the acute-phase reaction that follows brain ischemia? If so, does this modulation bear any impact on early stroke recovery? Are these effects dependent of the stroke subtype? We argue that demonstrating that high-dose UH decreases the acute-phase reaction that follows ischemic stroke and that the effect is positively associated with stroke recovery would support the design and funding of a clinical trial aimed at evaluating prospectively the therapeutic value of high-dose UH in patients with acute ischemic stroke. The identification of different inflammatory responses in patients with varied stroke subtypes would also assist in the selection of the best candidates to participate in such a trial.

2. Methods

All the patients with ischemic stroke that were admitted consecutively to our Stroke Unit from October 1992 to December 1998 and who were treated with high-dose sodium unfractionated heparin (UH) or aspirin within 72 h of stroke onset were eligible to participate in the study. Patients with diminished level of consciousness, active seizures, hemorrhagic stroke, transient ischemic attacks, recent ($<$1 month) history of myocardial infarction, sepsis, bacterial infection, malignancy, pregnancy, severe renal or hepatic impairment, collagen disease, thrombocytopenia, thrombocytosis, deep venous thrombosis or participation in a clinical trial were excluded. To further limit the potential confounding effect of systemic infections patients with admission body temperature $>$37.5°C were not included in the study. The treating physician determined the treatment modality depending on whether there was an embolic source or a high risk of recurrence or thrombus formation. Thus, treatment allocation was unblinded. At our unit, a decreased level of consciousness and the presence of active seizures are the main clinical features that determine exclusion from the use of UH as both conditions complicate the identification of treatment related side effects. Otherwise, the type of antithrombotic regime is selected based on the suspected stroke mechanism. Patients allocated to UH received the drug to achieve an activated partial thromboplastin time (aPTT) 1.5–2 times control values as soon as possible. Frequent aPTT monitoring are performed until targeted value are obtained. UH is maintained for 7 ± 2 days by pump-controlled Intravenous infusion of 1.000 units per hour without initial intravenous bolusing [14]. All patients allocated to aspirin received 300 mg/day of an enteric coated preparation. Regardless of treatment assignment, all patients received similar intensity of monitoring and care in aspects such as nutrition, hydration, blood glucose, blood pressure, temperature, infections and rehabilitation. The patients neurological condition was scored prior to treatment onset and at hospital discharge using the Mathew scale [15]. This scale evaluates neurological impairment and functional disability and has shown very good correlation with the Orgogozo scale, Scandinavian scale, NIH stroke scale and the Canadian scale [16]. Neurological recovery at hospital discharge was defined as complete if the Mathew scale score was 100 (normal=100). Neurological scoring was not assessed in this study at longer time intervals because many patients were transferred to other centers or were followed by their general practitioners. Patients allocated to UH or aspirin had a brain CT scan before treatment initiation, prior to hospital discharge, and whenever the patient's condition worsened. Cerebral or systemic bleeding that resulted in clinical worsening or that required blood transfusions were defined as serious. Recurrent stroke was defined as the presentation of new symptoms attributable to vascular territories different from the qualifying event, or to the same vascular territory if there was a clear-cut phase of stability between the two events. Diagnostic tests aimed at defining the cause of stroke were performed as appropriate in a tertiary hospital.

Acute-phase reactants assessed included the erythrocyte sedimentation rate (ESR) by the Westergren method, total leukocyte count ($\times10^9$/l), and percentage of polymorphonuclears (PMN, %), respectively [17,18]. These parameters were measured after treatment with UH or aspirin for at least 12–24 h. Other acute phase reactants such as fibrinogen and C reactive protein were not routinely measured.

The most likely stroke mechanism was classified as atheothrombotic, cardioembolic, undetermined, or lacunar according to standard criteria [19], In most instances, the stroke subtype was established at the end of the diagnostic work-up. For the purpose of this study the stroke subtypes were further dichotomized at the completion of the diagnostic work- up into lacunar or nonlacunar because reperfusion injury plays a different role in these groups [20], In short, lacunar infarction described patients with a classical lacunar syndrome if a brain CT scan or the MRI demonstrated normal findings or acute ischemic lesions of

Table 1
Main characteristics of the two antithrombotic regimes and stroke subtypes

	All strokes		Nonlacunar strokes		Lacunar strokes	
	Aspirin	Heparin	Aspirin	Heparin	Aspirin	Heparin
n	256	450	161	410	95	40
Age, years (mean±S.D.)	69.1±12.5	68.7±11.8	70.5±13.6	68.6±12.0	66.8±10.1	70.0±9.9
Male sex (%)	141 (55)	269 (60)	86 (53)	241 (59)	55 (58)	28 (70)
Atrial fibrillation (%)	46 (18)	123 (27)*	40 (25)	117 (29)	6 (6)	6 (15)
Hypertension (%)	116 (45)	194 (43)	78 (48)	180 (44)	38 (40)	14 (35)
Diabetes (%)	66 (26)	106 (24)	37 (23)	96 (23)	29 (31)	10 (25)
Coronary artery disease (%)	58 (23)	108 (24)	46 (29)	102 (25)	12 (13)	6 (15)

*, $P<0.01$.

less than 15 mm in maximal diameter located in deep anatomical structures, The classification of nonlacunar stroke included those patients with cardioembolic stroke, atherothrombotic stroke or stroke of undetermined cause. The prevalence of risk factors such as arterial hypertension, diabetes, atrial fibrillation, and coronary heart disease were assessed as previously described [18].

For statistical analyses parametric and nonparametric tests were performed as appropriate. ANCOVA models were used to analyze the independent contribution of the acute-phase reactants to the degree of neurological recovery at hospital discharge. Separate analyses were conducted to assess this question in the whole group of patients, and in those with nonlacunar and lacunar stroke, respectively. Variables that were entered in these models were antithrombotic therapy (aspirin versus UH), baseline Mathew score (continuous), treatment delay (h), and the interaction term between treatment type and treatment delay. Potential confounders that were included in the models were the incidence of early recurrent stroke and symptomatic bleeding,respectively. Explanatory variables without normal distribution were transformed into a logarithmic scale. Continuous data were expressed as mean S.D. and dichotomous data as proportions. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Description of the population and main clinical findings

A total of 706 patients were entered in the study including 450 patients treated with high-dose UH, and 256 treated with 300 mg/day aspirin. An additional group of 1200 patients were not included in the study because they had at least one of the exclusion criteria described in Methods. The main demographic characteristics of the patients and the prevalence of risk factors are shown in Table 1. With the exception of a greater prevalence of atrial fibrillation in patients that received UH there were no other significant differences between the two treatment groups. As depicted in Table 2, the delay to treatment onset was similar in both treatment groups as well as was the severity of neurological impairment at baseline in the total group of patients. Although patients with nonlacunar stroke treated with heparin were slightly less impaired at baseline than patients treated with aspirin a greater degree of recovery was observed in anticoagulated patients using ANCOVA and baseline impairment as covariate. Table 2 also highlights that the rate of death, stroke recurrence, and symptomatic bleeding observed in both treatment groups

Table 2
Main clinical findings in both treatments and stroke subtype groups[a]

	All strokes		Nonlacunlar strokes		Lacunar strokes	
	Aspirin	Heparin	Aspirin	Heparin	Aspirin	Heparin
n	256	450	161	410	95	40
Treatment delay (h)	24.2±15.7	22.5±15.9	23.2±14.7	22.4±15.9	25.9±17.2	24.0±16.0
Baseline Mathew	74.4±17.9	72.8±11.8	68.2±18.4*	71.8±16.0	84.7±11.2	82.7±8.2
Discharge Mathew	76.4±25.8*	79.3±21.4	69.4±28.4***	78.2±21.9	88.3±14.3**	91.1±7.8
Death	15 (5.8)	13 (2.8)	14 (8.7)	13 (3.1)	1 (1.0)	0 (0)
Serious bleeding	5 (1.9)	21 (4.6)	3 (1.8)	20 (4.8)	2 (2.1)	1 (2.5)
Recurrent stroke	6 (2.3)	15 (3.3)	4 (2.8)	15 (3.6)	2 (2.1)	0 (0)

[a] Comparisons refer to heparin versus aspirin. Discharge Mathew score is adjusted in both treatment groups for baseline Mathew using ANCOVA. *, $P<0.05$; **, $P<0.01$; ***, $P=0.0001$.

did not attain statistical significance. The length of hospital stay was slightly longer in patients who received UH than in those treated with aspirin (12.7±7.2 vs. 11.3±6.4 days, $P<0.01$).

3.2. Relationship between acute-phase reactants and antithrombotic regimen. clinical implications

The total number of leukocytes, percent of PMN, and ESR, were significantly higher in patients who received aspirin than in those who were treated with UH. As shown in Table 3, although these differences were observed in the whole group further analyses disclosed there were only significant in the group of patients with nonlacunar stroke, Simple linear regression showed that the total number of leukocytes, percent of PMN, and ESR were correlated with a worst neurological score at first examination (all P values of 0.0001). Moreover, the acute phase reaction was statistically significant lower in patients with complete recovery than in uncompletely recovered patients, as shown in Table 4. Again, these differences only attained statistical significance in the group of patients with non-lacunar stroke.

3.3. Independent predictors of neurological severity at hospital discharge

Separate ANCOVA models were performed to evaluate the extent of neurological recovery at hospital discharge in patients with nonlacunar or lacunar stroke. As shown in Table 5, independent outcome predictors for patients with lacunar and nonlacunar stroke included neurological score at baseline, stroke recurrence, and serious bleeding. Both models disclosed a positive association with the use of UH and complete recovery. However, a greater β coefficient indicating a stronger effect was observed in the group of patients with nonlacunar stroke stroke. In this group, a negative association was also found for the total leukocyte count (continuous), and the delay to treatment onset (continuous). In none of these models, did the percent of PMN or the ESR remain associated to stroke recovery at a statistically significant level.

Table 4
Acute-phase reactants and clinical recovery

	Functional recovery	
	Incomplete	Complete
All strokes		
Total leukocytes ($\times 10^9$/l)*	8.4±4.0	7.2±2.3
PMN[a]* (%)	61.6±19.0	56.6±13.1
ESR[a]***	24.3±22.2	15.7±17.2
Nonlacunar strokes		
Total leukocytes ($\times 10^9$/l)***	8.5±3.0	7.2±2.3
PMN* (%)	62.8±19.3	57.0±12.8
ESR**	25.4±22.9	16.0±18.2
Lacunar strokes		
Total leukocytes ($\times 10^9$/l)	7.9±7.0	7.3±2.3
PMN (%)	56.1±16.6	55.2±15.3
ESR	20.0±18.2	14.3±12.6

[a] PMN, polymorphonuclears; ESR, erythrocyte sedimentation rate. *, $P<0.01$; **, $P<0.001$; ***, $P=0.0001$.

Table 5
Independent predictors of Mathew score at hospital discharge

Variable	β	†	95% Cl	
Model A. Lacunar stroke patients				
Heparin (versus aspirin)	3.9	3.0	1.3	6.6
Baseline Mathew (continuous)	0.8	15.2	0.7	0.9
Recurrent stroke (versus no)	−31.7	−5.8	−42.4	−21.0
Serious bleeding (versus no)	−19.4	−4.4	−28.1	−10.7
Adjusted $R^2=0.70$				
Model B. Nonlacunar stroke patients				
Heparin (versus aspirin)	13.0	4.2	6.9	19.0
Baseline Mathew (continuous)	0.9	19.5	0.8	1.0
Recurrent stroke (versus no)	−18.7	−4.5	−26.8	−10.6
Serious bleeding (versus no)	−15.2	−3.8	−22.9	−7.5
Total leukocyte count (continuous)	−1.0	−3.9	−1.5	−0.5
Treatment delay in h (continuous)	−0.2	2.6	0.0	0.4
Adjusted $R^2=0.51$				

4. Discussion

Prompted by recent studies suggesting the antiinflammatory capacity of UH in several systemic conditions and in animal models of focal ischemia–reperfusion [8–12,21–

Table 3
Total leukocyte count, percentage PMN, and ESR according to treatment and stroke subtype

	All strokes		Nonlacunlar strokes		Lacunar strokes	
	Aspirin	Heparin	Aspirin	Heparin	Aspirin	Heparin
n	256	450	161	410	95	40
Total leukocytes ($\times 10^9$/l)	8.6±3.2**	8.0±4.1	9.4±3.5***	7.9±2.5	7.3±1.9	9.0±11.4
PMN[a] (%)	61.4±19.0	60.5±17.9	65.3±19.2**	60.7±18.2	54.9±16.8	58.6±14.8
ESR[a]	25.2±22.9*	21.7±20.9	28.2±24.5**	22.3±21.4	20.1±19.0	16.6±13.5

[a] PMN, polymorphonuclears; ESR, erythrocyte sedimentation rate. *, $P<0.05$; **, $P<0.01$; ***, $P=0.001$.

25], we compared the magnitude of the acute-phase response that followed ischemic stroke in 706 patients treated with either high dose UH or 300 mg/day of aspirin as soon as they were brought to our attention at the stroke unit. We hypothesized that the release of acute-phase reactants would be less marked in the group of anticoagulated patients. The study was also aimed at determining whether a decrease in the acute-phase response bore any prognostic implications at hospital discharge [18].

Segregated comparisons were performed for patients with nonlacunar or lacunar strokes because, in all likelihood, inflammation and reperfusion damage play a secondary role in the latter stroke subtype.

The results of the study confirmed our hypotheses as we observed that the total number of leukocytes, percent of PMN, and ESR were significantly less pronounced in the group of patients treated with UH. Analyses performed for each stroke subtype further disclosed that differences were restricted to patients with nonlacunar stroke, More importantly, a lower acute-phase response in this stroke subgroup was independently associated with early complete recovery. Other independent prognostic factor restricted to nonlacunar stroke included a shorter delay to treatment onset. Notably, these independent associations with stroke outcome remained statistically significant despite the inclusion in the models of powerful negative outcome predictors such as stroke recurrence and bleeding complications. These findings are in discrepancy with most previous antithrombotic trials which consistently failed to show a net clinical benefit for early anticoagulation in acute ischemic stroke. We argue that the lack of agreement could mainly be due to the lower dose of UH selected in previous clinical trials, the excessive delay to treatment onset, and the inclusion of a significant proportion of subjects with lacunar stroke.

Our results are better understood in light of recent advances regarding the mechanisms of action of UH. In addition to its well described antithrombotic effects UH also inhibits leukocyte rolling on the vessel wall [21], due to its ability to block selectins on leukocyte and platelets [23]. Although the expression of adhesion molecules seems to be unaffected, UH impairs the cell-adhesive interactions between leukocytes and endothelial cells [24]. Recently, in vitro studies have also shown than UH attenuates the increase in inducible nitric oxide synthase (iNOS) and NO release after cytokine activation [25]. These findings suggest that UH influences the cytokine–membrane interaction or the early steps of signal transduction in inflamed endothelial cells. Importantly, all these studies have emphasized that these antiinflammatory properties are only seen if UH is administered at full doses and very soon after the onset of symptoms. In support of these observations we recently found that the administration of high-dose UH to patients with cardioembolic stroke was most effective the sooner the drug was administered [26].

Several characteristics of the present study deserve attention. We found a positive association between the initial severity of symptoms and a greater acute-phase response. Therefore, it could be suggested that the acute-phase response that was measured merely reflected the extent of ischemic brain injury. Previous studies also found that the magnitude of the inflammatory response was greater in stroke patients with larger lesions [27,28], and in those with incomplete recovery [17,18]. Although an elevation of the ESR and total leukocyte count is a nonspecific reaction to injury, previous work has shown a very good correlation between these parameters and the release of a variety of cytokines in patients with acute stroke [17,18]. In line with these observations, the inhibition of leukocytes with antineutrophil antibodies was found associated with a reduction of the infarct size in murine models of icschemia–reperfusion [28,29]. However, as our multivariate analysis was adjusted for the severity of stroke on admission, the independent association that was found between the acute-phase response and clinical outcome stressed the contribution of inflammatory processes to the final fate of ischemic brain injury. Of course, this association did not preclude that the initial destruction of brain tissue also contributed to the initial peak of inflammatory markers under ischemic conditions.

The major limitation of the study is that treatment allocation was not randomly assigned. Therefore, we are very cautious when extrapolating the prognostic implications of these results. Nevertheless, we emphasize that both treatment groups evidenced similar neurological impairment at hospital admission. The time to treatment onset was also similar in both groups and the type of care given to all patients was not influenced by the antithrombotic regime. However, as a result of the unblinded nature of the study we cannot totally exclude the possibility that the anticoagulated patients were more carefully monitored. Obviously, the only way to circumvent such a limitation is by performing a new clinical trial. Based on our results, we believe that such a clinical trial is fully warranted [30]. Its design has been recently completed under the acronym RAPID (rapid anticoagulation prevents ischemic damage) and, hopefully, it will be carried out in approximately forty centers from seven European countries. Bringing together previous experimental data and the findings discussed in this study, patients with lacunar stroke will not be included, and the delay to treatment onset will be minimized to optimize the ‘neuroprotective’ effects of high-dose UH.

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3. Unfractionated heparin is associated with a lower rise of serum vascular cell adhesion molecule-1 in acute ischemic stroke patients.

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Unfractionated heparin is associated with a lower rise of serum vascular cell adhesion molecule-1 in acute ischemic stroke patients

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Abstract

We sought to assess the anti-inflammatory properties of unfractionated heparin (UFH) in patients with ischemic stroke treated within 24 h from the onset of symptoms. We studied prospectively 167 patients that received 1000 IU/h intravenous UFH ($n = 70$) or 300 mg oral aspirin ($n = 97$) at a mean treatment delay of 6.7 h. Repeated plasma levels of interleukin (IL)-6, IL-10, IL-4, tumor necrosis factor (TNF)-α, soluble intercellular adhesion molecule-1 (sICAM-1), and soluble vascular cell adhesion molecule-1 (sVCAM-1) were compared in both groups using multivariate analyses. Whereas TNF-α and sICAM-1 decreased at 48 h, IL-6, IL-4, and sVCAM-1 increased compared with baseline values ($P < 0.01$). The rise of sVCAM-1 levels at 48 h was significantly lower in patients treated with UFH ($P = 0.017$) and a two-fold increase of baseline sVCAM-1 was an independent predictor of poor outcome (odds ratio, 2.19, 1.1–4.39). These results suggest that adjusted high-dose UFH has anti-inflammatory effects which might improve recovery if administered early after stroke onset.

Keywords: Cerebrovascular disease; Heparin; Aspirin; Cytokines; Vascular cell adhesion molecule-1; Inflammation; Outcome

Inflammation plays a pivotal role in focal brain ischemia [17]. The hallmark of the inflammatory reaction is the presence of leukocytes in the ischemic zone [9]. Leukocyte infiltration is preceded by the expression of cytokines and endothelial cell adhesion molecules, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). These molecules propitiate ischemic damage by vascular plugging, conversion of the endothelium to a prothrombotic state, rheologic effects, and release of oxygen radicals and cytotoxic products [13]. As the intensity of these effects in stroke correlate with the severity of symptoms and extent of the infarction [18], therapeutic interventions aimed at decreasing proinflammatory effects might result in a better outcome in this population [8]. Human and experimental studies have demonstrated binding of unfractionated heparin (UFH) to leukocitary integrins involved in coagulation, inflammation, and cell proliferation processes [19]. In the rodent model of focal brain ischemia described by Yanaka et al., UFH inhibited leukocyte accumulation, reduced infarction size, and improved neurological function in a time- and dose-dependent manner [21]. Thus, we assessed the concentration of several cytokines and soluble cell adhesion molecules in stroke patients who received high-dose UFH or aspirin to elucidate the relative anti-inflammatory properties of these compounds. A group of 167 consecutive patients (58% males; mean age, 67.7 ± 9.8) with first-ever ischemic stroke was included in the study according to a protocol approved by the local Ethics Committee of participating centers. Patients received high-dose intravenous (i.v.) UFH ($n = 70$) or 300 mg oral aspirin ($n = 97$) within 24 h from the onset of symptoms. Exclusion criteria included history of recent infection, inflammatory condition, cancer, hematological disease, severe renal or liver failure, or participation in investigation therapeutic trials. The type of stroke was classified according to TOAST criteria [1]. A brain

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computed tomography (CT) scan was mandatory prior to treatment onset and on days 4–7. All CT scans were reviewed by blinded observers. The severity of stroke was repeatedly scored using the Canadian Stroke Scale (CSS). Outcome was also assessed at 6 months after stroke onset using the Barthel Index (BI). Poor outcome was defined as death ($n = 8$) or BI score <85 ($n = 60$) at the end of follow-up [12]. According to the decision of the treating physician, patients were allocated to receive UFH 1000 IU/h i.v. or aspirin 300 mg/day. In the UHF group, monitoring of the activated partial thromboplastine time (APTT) was initiated 6 ± 2 h after treatment onset and repeated as needed to achieve an APTT 1.5–2 times control values.

Blood samples were collected before the onset of antithrombotic therapy (less than 24 h from symptom onset), and after 48 h by personnel unaware of the clinical and therapeutic data in tubes with potassium edetate, centrifuged at $2000 \times g$ for 10 min., immediately frozen at −80 °C, and stored until analysis. IL-6, IL-10, IL-4, TNF-α, soluble ICAM-1 (sICAM-1), and soluble VCAM-1 (sVCAM-1) concentrations in plasma were measured in the same laboratory with commercially available quantitative sandwich enzyme-linked immunoadsorbent assays (Quantikine, R&D System, Minneapolis, MN). Cytokine and soluble adhesion cell molecule levels are expressed as geometric means (95% CI). Medians (quartiles) were used when continuous variables were not normally distributed (Shappiro–Wilks test) and log transformation could not be performed. Between-group comparisons of cytokine and soluble adhesion cell molecule levels were performed using parametric tests after log transformation of variables without normal distribution. Outcome was evaluated using stepwise logistic regression analyses in two separate models. The first model assessed the independent contribution of clinicoradiological variables. The second model assessed the independent contribution of cytokine and soluble adhesion cell molecule levels adjusting for the variables with statistical significance encountered in the first model. Whether UFH and aspirin influenced the time course of individual cytokine and soluble adhesion cell molecule levels was evaluated using multiple linear regression analyses.

Table 1
Infarction volume and clinical outcome in patients treated with aspirin or UFH

	Aspirin group[a] $n = 70$	UFH group[a] $n = 97$
CSS		
Baseline	5.5 (3.0–7.5)	6.0 (3.9–8.0)
48 h[b]	3.5 (2.0–7.0)	7.0 (4.5–8.5)
7 days[b]	4.5 (3.0–7.0)	8.0 (5.5–9.5)
3 months[b]	7.0 (5.0–9.0)	9.0 (6.9–10.0)
6 months[b]	7.0 (5.0–9.5)	10.0 (7.0–10.0)
Infarct volume (cc)[b]	26.5 (6.5–75.8)	11.8 (4.0–24.1)

[a] Median (quartiles).
[b] $P < 0.001$.

Table 2
Correlation of baseline soluble adhesion molecule and cytokine levels with the initial findings on brain CT scan[a]

	Presence of early CT signs of infarction $n = 79$	Absence of early CT scan signs of infarction $n = 88$
IL-6 (pg/ml)[b]	17.9 (14.2–22.5)	9.2 (7.41–1.2)
IL-4 (pg/ml)	19.4 (18.2–20.6)	20.2 (19.1–21.3)
IL-10 (pg/ml)	5.5 (5.0–6.0)	5.7 (5.2–6.1)
TNF-α (pg/ml)[c]	17.8 (16.1–19.7)	14.8 (13.5–16.1)
sICAM-1 (ng/ml)[b]	357.0 (297.8–427.8)	214.8 (197.8–233.2)
sVCAM-1 (ng/ml)	588.9 (541.7–640.1)	560.9 (516.2–609.4)

[a] Numbers are geometric means (95% CI).
[b] $P < 0.0001$, using Wilconson rank-sum test.
[c] $P = 0.007$, using Wilconson rank-sum test.

There were no differences in neurological severity at baseline between the two antithrombotic groups ($P = 0.27$), as shown in Table 1. Variables that were independently associated with BI score of <85 included: baseline CSS score—one point decrease (odds ratio (OR), 1.66; 1.31–2.11; $P < 0.001$); baseline CT scan—normal/abnormal (OR, 11.30; 3.99–32.46; $P < 0.001$); and axillary temperature—1 °C increase (OR, 3.50; 1.72–7.15; $P < 0.001$). A trend was also observed for mean arterial pressure—5 mmHg increase (OR, 1.02; 1.0–1.05; $P = 0.08$). Mean values at 48 h of TNF-α and sICAM-1 were significantly lower than at baseline (both $P < 0.001$) (Fig. 1). Contrarily, mean values of IL-6, IL-4, and sVCAM-1 were significantly higher at follow-up (all $P < 0.01$). IL-10 was the only cytokine that did not experience significant longitudinal changes ($P = 0.40$). Baseline levels of IL-6, TNF-α, and sICAM-1 were higher in patients that showed early CT signs of cerebral infarction, as described in Table 2. Other inflammatory markers were not found to be associated with the initial CT findings. sVCAM-1 at 48 h was the only inflammatory marker independently associated with BI score at 6 months (Table 3). After adjusting for baseline inflammatory values, baseline

Table 3
Independent clinical, hemodynamic, radiological, and inflammatory variables associated with BI score of <85

Variable[a]	OR	95% CI	P
Two-fold increase of sVCAM-1 at 48 h	2.19	1.1–4.39	0.02
One point decrease of CSS score at baseline	1.66	1.31–2.12	<0.001
Early CT signs of infarction	8.30	2.8–24.64	<0.001
1° C increase in axillary temperature	3.23	1.58–6.6	0.001
5 mmHg increase in mean arterial pressure	1.16	1.01–1.33	0.03

[a] CSS, Canadian Stroke Scale.

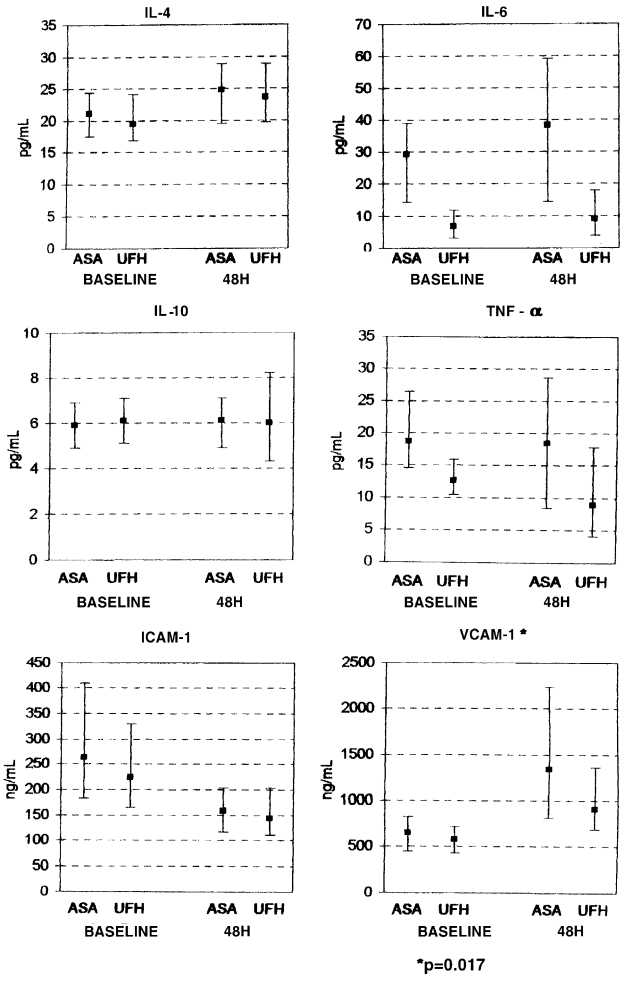

Fig. 1. Longitudinal time course of inflammatory concentration levels (medians and quartiles) in patients with ischemic stroke treated with UFH or aspirin.

CSS, baseline CT scan, and mean arterial pressure values of sVCAM-1 were on average 1.24 (95% CI, 1.04–1.49; $P = 0.017$) higher in the aspirin group that in the heparin group. The time course of the remaining biological parameters did not differ between the two antithrombotic groups (Fig. 1).

This study confirms the previously reported marked longitudinal changes in soluble cell adhesion molecule concentrations in patients with acute ischemic stroke [3]. Regardless of the initial severity of neurological symptoms and admission CT findings, we found an inverse relationship between increased sVCAM-1 at follow-up and the chances of functional recovery. Namely, a two-fold increment of baseline sVCAM-1 levels at 48 h doubled the odds of poor outcome. The time course of IL-6, IL-4, IL-10, TNF-α, and sICAM-1 was not associated with patient outcome at 6 months, and the concentration of these compounds did not differ between patients treated with UFH or aspirin. Contrarily, the rise of sVCAM-1 at 48 h was less pronounced in patients treated with high-dose UFH than in patients treated with aspirin. Although we cannot exclude that UFH and aspirin modulated other inflammatory markers, sVCAM-1 emerged as the only inflammatory compound associated with functional outcome. We believe that the results reflect true anti-inflammatory differences between UFH and aspirin because the patients were assessed prospectively, UFH was used according to previously specified guidelines, and the inflammatory markers were measured by personnel unaware of the clinical and therapeutic data. Further, the statistical methods took careful consideration of potential confounders such as type of stroke, initial neurological impairment, early CT findings, delay to treatment, incident infections, and magnitude of the acute-phase response.

The relevance of the therapeutic window may also be important when antithrombotic agents are administered [5]. Experimental models of focal brain ischemia in the rat emphasized the strong relationship between treatment delay, treatment dose, extent of neurological recovery, and infarction size [21]. A recent clinical study further stressed the relevance of early anticoagulation in stroke, as the odds of complete recovery almost doubled in patients anticoagulated within 6 h of onset compared with those anticoagulated at longer delays [7]. Based on our current findings, it could be argued that early anticoagulation allows a more effective modulation of sVCAM-1-mediated brain tissue damage.

VCAM-1 is an adhesion factor expressed following endothelial activation by cytokines that selectively binds mononuclear cells such as lymphocytes and monocytes [20]. Resting endothelial cell express minimal or undetectable levels of VCAM-1. Detectable surface protein is found between 2 and 4 h after cytokine exposure, reaching maximum levels after 12 h and lasting for up to 72 h. VCAM-1 contribute to firm adhesion and transendothelial migration of mononuclear cells when their counter-receptors are in a high affinity state. These counter-receptors are expressed by lymphocytes, monocytes, eosinophils and basophils, but not by circulating neutrophils [20]. Intense expression of VCAM-1 by astrocytes and endothelial cells from the infarcted tissue, but not from the non-infarcted areas, has been observed [4]. Raised levels of VCAM-1 in stroke survivors suggest that VCAM-1 might be part of the pathophysiological response to brain tissue repair [4].

sVCAM-1 could facilitate brain damage through the expression of tissue factor (TF) as ligation of monocytic integrins to endothelial VCAM-1 induces TF expression [14]. TF is diffusely expressed in astrocytes and arachnoid meningeal cells [10], and it represents the primary cellular initiator of the coagulation protease cascade [2]. UFH abrogates the endotoxin-induced increase in TF-positive monocytes in vivo [15], and it also increases plasma levels of TF pathway inhibitor [16]. The inhibitory effect of UFH on TF expression is mediated by the ability of UFH to reduce the expression of IL-1β, TNF-α, IL-6, and L- and P-selectins which in turn enhance TF production [11]. Nevertheless, further studies will be needed to clarify the molecular

mechanisms by which UFH decreases sVCAM-1 in acute ischemic stroke.

In summary, the study shows that UFH was associated with a smaller increase of sVCAM-1 than aspirin, and that this effect was related to greater clinical recovery at 6 months in patients with acute ischemic stroke. To comply with the rules of evidence-based medicine, the clinical value of adjusted-dose i.v. UFH in patients with acute ischemic stroke should be assessed in a randomized clinical trial [6].

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4. The Rapid Anticoagulation Prevents Ischemic Damage Study in Acute Stroke – Final Results from the Writing Committee.

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The Rapid Anticoagulation Prevents Ischemic Damage Study in Acute Stroke – Final Results from the Writing Committee

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In the rat ischemic brain, unfractionated heparin (UH) is neuroprotective [1], although its clinical value in acute stroke is still a matter of controversy [2–10]. The Rapid Anticoagulation Prevents Ischemic Damage (RAPID) Trial compared the value of aspirin and UH in patients with suspected nonlacunar ischemic stroke and symptoms lasting less than 12 h using a parallel group, randomized, open, blinded endpoint assessment (PROBE) design. A brain CT scan was mandatory at baseline to exclude any amount of blood, and patients or proxies signed an informed written consent. UH was started with an intravenous bolus of 40 IU/kg of UH followed by a weight-adjusted continuous infusion of 12 IU/kg/h aimed to achieve UH plasma levels of 0.3–0.5 U/ml as soon as possible. Control of UH therapy was assessed with aPTT ratios (patient aPTT/control aPTT). Six hours after treatment onset, or after any dose adjustment, an aPTT was obtained. Otherwise, aPTTs were ordered every 12 h. Local laboratories were requested at study onset to calibrate the aPTT ratio to their corresponding UH levels. Aspirin (300 mg/day) was given orally or by nasogastric tube. The study treatment was maintained for 6 ± 1 days in stroke units of tertiary hospitals.

The primary endpoint of the RAPID study was the rate of favorable outcome defined as a modified Rankin scale ≤ 2 at day 90 ± 14 days. The modified Rankin scale was centrally assessed by telephone interview by investigators blind to treatment allocation. The main secondary endpoints included the single and combined incidence of recurrent stroke (appearance of new symptoms), worsening stroke (at least 4 points change in NIHSS score), symptomatic intracerebral hemorrhage, systemic bleeding, deep venous thrombosis, pulmonary embolism and death at day 90. A Steering Committee and a DSMB directed and supervised the study. All centers had the approval of an independent ethics committee. For sample size calculations, 592 patients per treatment group were required (α 0.05%, β 80%) to detect or disprove an absolute difference of about 8% in favorable outcome. Parametric and nonparametric tests were used as appropriate. Thirty months after the onset of the study, recruitment was closed after 67 patients had been recruited, emphasizing the difficulties of academic trials to recruit centers and patients without adequate funding, regardless of the clinical relevance of the question addressed.

As illustrated in table 1, there were close similarities between the 2 treatment groups, although a higher proportion of patients allocated to UH had large infarctions at baseline CT scan. As shown

Table 1. Main characteristics of the RAPID study

	Treatment arm		p
	ASA (n = 35)	UH (n = 32)	
Demographics and risk factors			
Males	23 (66)	17 (53)	0.29
History of hypertension	20 (57)	18 (56)	0.94
History of diabetes	8 (23)	10 (31)	0.43
Active smoking	8 (23)	6 (19)	0.68
High cholesterol	10 (29)	6 (19)	0.34
Previous stroke	2 (6)	0 (0)	0.17
Atrial fibrillation	10 (29)	13 (41)	0.29
Coronary heart disease	5 (14)	4 (13)	0.83
Aspirin at clinical onset	6 (18)	8 (26)	0.42
Admission parameters, mean ± SD			
Age, years	69.4 ± 10.2	72.2 ± 9.6	0.27
SBP, mm Hg	159.0 ± 25.3	151.0 ± 27.0	0.21
DBP, mm Hg	86.0 ± 14.4	82.2 ± 14.7	0.29
Platelet count, × 1,000/mm^3	215.7 ± 63.9	225.1 ± 63.4	0.54
Leukocyte count, × 1,000/mm^3	8.5 ± 3.2	9.5 ± 3.1	0.18
Glucose, mg/dl	143.6 ± 46.6	138.7 ± 52.9	0.78
Uric acid, mg/dl	5.4 ± 1.9	5.3 ± 1.6	0.82
Creatinine, mg/dl	1.05 ± 0.2	1.04 ± 0.2	0.90
Qualifying event			
Stroke subtype			0.41
ATH	10 (29)	6 (19)	
EMB	13 (37)	18 (56)	
LAC	1 (3)	–	
Unknown	10 (29)	8 (25)	
Other	1 (3)	–	
Baseline NIHSS, mean ± SD	13.2 ± 5.8	13.4 ± 5.9	0.86
Abnormal baseline CT scan	21 (60)	27 (84)	0.02
Lesion >1/3 of arterial territory	5 (14)	12 (38)	0.02
Delay to first dose			
mean ± SD, min	388 ± 207	450 ± 204	0.23

Figures in parentheses indicate percentages.

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Table 2. Primary and secondary endpoints of the RAPID study

	Treatment arm		p	Odds ratio (95% CI)
	ASA (n = 35)	UH (n = 32)		
Rankin 0–2 at 3 months	19 (54.3)	13 (40.6)	0.26	1.74 (0.7–4.6)
NIHSS at day 7, mean ± SD	12.3 ± 11.7	12.4 ± 11.6	0.97	
NIHSS <1 at day 90 ± 14	9 (25.7)	8 (25.0)	0.94	1.04 (0.34–3.13)
Ischemic stroke recurrence	3 (8.6)	0 (0)	0.09	0.13 (0.01–1.39)
Any death, at day 90	4 (11.4)	6 (18.8)	0.40	0.56 (0.14–2.2)
Vascular death, at day 90	3 (8.6)	4 (12.5)	0.60	
Changed outcome at day 7			0.46	
Improved	19 (54.3)	14 (43.8)		1.53 (0.58–4.01)
Unchanged	8 (22.9)	12 (37.5)		0.49 (0.17–1.43)
Worse	8 (22.9)	6 (18.8)		1.28 (0.39–4.21)
Hemorrhagic worsening	3 (8.6)	2 (6.3)	0.71	1.41 (0.2–9.0)
Ischemic worsening	7 (20.0)	8 (25.0)	0.62	0.75 (0.4–2.4)
DVT or pulmonary embolism	0	0		
Platelet decrease >40%	0	1 (3.1)	0.29	
Death, recurrence or bleeding at day 7	5 (14.3)	4 (12.5)	0.83	1.17 (0.28–4.79)

Numbers (proportions), unless stated otherwise.

Worsening defines a follow-up NIHSS score of 4 or more additional points than baseline NIHSS score. In the ASA and UH columns, figures in parentheses indicate percentages.

in table 2, a trend to a lower recurrence rate in patients allocated UH was the only outcome measure difference between the 2 groups. The main treatment effect was not confounded by imbalanced longitudinal values of blood pressure, body temperature or glucose levels (data not shown). Targeted UH levels were achieved within 24 h of treatment in 41% of patients allocated to UH. In 53%, UH levels were insufficient, and in 6%, excessive. Ischemic stroke worsening (n = 8) was associated with lower UH levels than clinically stable strokes (n = 24) at day 1 (0.13 ± 0.13 vs. 0.31 ± 0.16, $p < 0.01$) and at day 2 (0.17 ± 0.13 vs. 0.35 ± 0.11, $p < 0.01$). Hemorrhagic worsening (n = 2) was associated with higher levels of UH than bland infarctions at day 1 (0.13 ± 0.13 vs. 0.31 ± 0.16, $p < 0.01$) and at day 2 (0.57 ± 0.34 versus 0.24 ± 0.14, $p < 0.01$). Unlike in previous trials of fixed, unmonitored doses of UH [5], in the RAPID study UH was at least as safe as ASA, although the study cannot exclude a theoretical 9-fold excess of hemorrhagic worsening. Nevertheless, the RAPID study suggests that the hemorrhagic risk can be diminished by close monitoring and adjustment of the aPTT. Worsening ischemic stroke was associated with UH levels lower than 0.3 U/ml, encountered in more than half of the patients during the first day of treatment. Thus, a larger benefit could have been achieved if the targeted values had been reached earlier.

The RAPID study is not adequately powered but still represents the largest trial comparing the risk benefit of ASA or high-dose weight-adjusted UH in acute ischemic stroke. Based on our findings, absence of evidence should not be equated to evidence of absence. Contrarily, we advocate the funding of a further clinical trial assessing in 1,184 patients with acute stroke whether early administration of weight-adjusted UH is neuroprotective. Meanwhile, the role of UH is an open issue.

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Microsurgical Anatomical Landmarks Associated with High Bifurcation Carotid Artery Surgery and Related to Hypoglossal Nerve

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Introduction

The hypoglossal nerve (HN) governs motor supply of the tongue. Injuries to HN, such as a complication of carotid artery surgery, occur in 2–17% of cases [1, 2]. When HN needs to be mobilized anterosuperiorly at the level of a high carotid bifurcation (CB), in order to gain a wide enough exposure, the risk of injury may be immense [3, 4]. In this anatomical study, we performed cadaveric neck dissections to assess the normal and the variated course of the nerve, and to display its relationships with neighboring anatomical structures.

Materials and Methods

The extracranial part of the HN was studied on 10 formalin-fixed adult cadaver heads (20 sides). The specimens were obtained after routine autopsy procedures had been performed, and were embalmed in 10% formaldehyde solution. The internal carotid arteries (ICAs), vertebral arteries and internal jugular veins (IJVs) were dissected, cannulated and irrigated with saline solution. The vascular structures were perfused with colored latex in 8 specimens, and with colored silicon in the remaining 2 specimens in order to facilitate their definition. Cadaveric heads were examined under 3–10× magnification using the Opmi-Zeiss surgical microscope. Measurements were calculated using 8 inch/200 mm electronic digital calipers (Marathon, Richmand Hill, Canada). The entire neck was dissected and measurements taken: the distance between the HN and the tendon of the digastric muscle, the distance from the origin of the occipital artery (OA) to the CB, the distance from the origin of the OA to the point where the HN crosses the OA, and the total lengths of the HN, hypoglossal loop and CB were measured (table 1).

Results

The extracranial part of the HN can be divided into 3 parts: descendens, transverse and lingual hypoglossus. The descendens hypoglossus originates at the external exit of the hypoglossal canal, at which point the HN crosses medially to the IJV, then posteromedially as far as the ICA. The HN then courses laterally and descends beyond the posterior aspect of the vagal nerve. The HN can usually be clearly identified, especially when following the ansa cervicalis superiorly, where it connects to the HN [5, 6].

We have described a new 'hypoglossal' triangle in the anterior part of the neck, which is bordered laterally by the descendens hypoglossus, inferiorly by the transverse hypoglossus and superiorly by the inferior border of the stylohyoid muscle (fig. 1). This triangle always included segments of the OA, ICA and external carotid artery (ECA). The HN becomes more superficial, 3.1–7.1 mm (mean 4.77 mm) inferior to the tendon of the posterior belly of the digastric muscle (fig. 2). An interesting observation, which was present in all specimens, was a point where HN crossed closely over OA. This crossing point was 7.1–9.4 mm (mean 8.27 mm) superior to the emergence of the OA from the ECA in all cadavers. At the level of the origin of the OA, the HN forms a loop and passes anteriorly along the lateral surface of both the ICA and the ECA, branching to become the superior root of the ansa cervicalis (fig. 1). The distance between the hypoglossal loop and the CB were measured: 14.5–25.2 mm (mean 19.24 mm). The OA arose from the posterior surface of the ECA, 6–9.2 mm (mean 7.25 mm) above the CB. After crossing over the OA, the transverse part of the HN begins and follows the anterolateral aspect of the ICA and ECA in every plane from the bifurcation to the inferior aspect of the digastric tendon. The ICA can be divided into three zones, as described by Hans et al. [4]. Fourteen of our 20 specimens (70%) could be defined as zone I, meaning extension from the CB to the upper margin of the third cervical vertebrae. Seven of our 20 specimens (30%) were zone II, the bifurcation extending from the lower margin of the second, to the lower margin of the first cervical vertebrae. There were no zone

IV. RESUMEN DE LOS RESULTADOS

1. Búsqueda de la dosis apropiada de HNF.

En un primer grupo de ratas evaluamos la farmacocinética de la HNF al administrar una dosis única intravenosa de 300 UI/kg. Esta dosis proporcionaba unos niveles plasmáticos del fármaco que desaparecían completamente a las 3 horas del tratamiento (Fig 1).

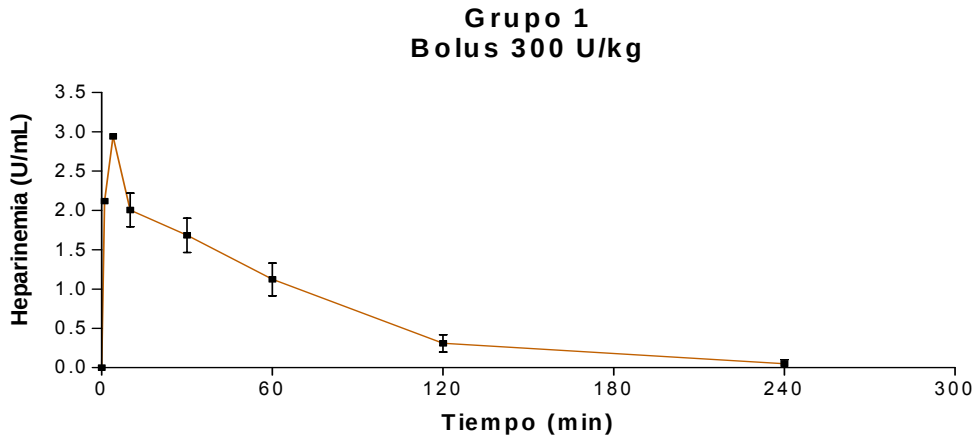

Fig. 1. Farmacocinética de la HNF tras un bolus de 300 UI/kg.

En otros grupos de ratas se administraron bolus de 300, 200 o 100 UI/kg seguidos de una perfusión intraperitoneal durante 24 horas a una dosis de 30, 50 o 70 UI/kg/hora. El régimen de tratamiento que cumplía mejor los requisitos de mantener una heparinemia constante de 0.3 a 0.6 U/mL fue un bolus inicial de 200 U/kg seguido de una perfusión intraperitoneal de 70 U/kg/h. Este fue el régimen elegido para el tratamiento de las ratas en los experimentos posteriores. A pesar del decremento en los niveles de heparinemia entre las 2 y las 6 horas, se descartó iniciar el tratamiento con un bolus de 300 U/kg, ya que se asociaba a complicaciones hemorrágicas. De la misma manera el bolus inicial de 100 U/kg provocaba niveles más prolongados de heparinemia infraterapéutica. Se administró la dosis elegida a ratas sometidas a isquemia cerebral focal y la heparinemia media fue de 0.46 ± 0.19 U/mL, estable en el tiempo y alcanzado la heparinemia objetivo (0.3-0.6 U/mL).

2. Efecto neuroprotector de la HNF.

Sometimos a un grupo de 26 ratas a isquemia cerebral focal transitoria de 1 hora de duración (figura 2), 13 tratadas con HNF y 13 con suero salino. El tratamiento se inició 3 horas después del inicio de la isquemia (a las 2 horas del inicio de la reperfusión). El efecto anticoagulante de la HNF se pudo comprobar a las 24 horas del inicio de la isquemia, mediante el TTPA. Las ratas tratadas con salino tuvieron un TTPA de 18.68 ± 2.68 segundos, mientras que en las tratadas con HNF fue 37.22 ± 8.45 segundos (figura 3). Durante la inducción de la isquemia y la administración del tratamiento, los valores de presión arterial y temperatura corporal fueron similares en los 2 grupos (tabla).

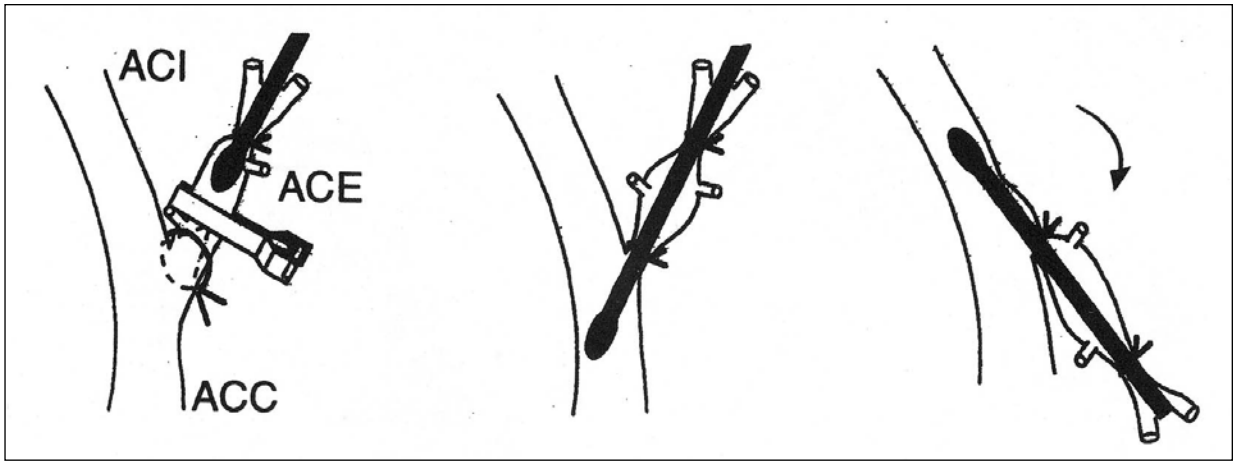

Fig. 2. Esquema del modelo de isquemia-reperfusión cerebral focal mediante oclusión endoluminal de la arteria cerebral media utilizado en nuestros experimentos.

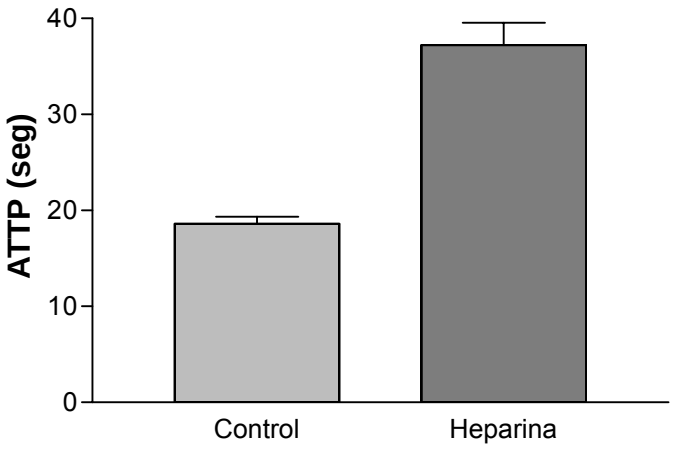

Fig. 3. Diferencias en el TTPA entre el grupo de ratas tratadas con placebo y con HNF.

	Presión	Temperatura	Peso
Grupo Control	87.99 ± 8.07	36.71 ± 0.17	307.2 ± 24.6
Grupo Tratado	89.58 ± 8.56	36.64 ± 0.24	291.5 ± 12.8
	ns	ns	ns

Tabla. Valores de presión arterial media (mm Hg) y temperatura media (ºC) de los animales durante la intervención, así como del peso (gr) medido el mismo día.

A las 24 horas del inicio de la isquemia se eutanasió a los animales. No se observaron hemorragias sistémicas o cerebrales. Los volúmenes de infarto cerebral medidos por TTC fueron 146.5 ± 35.66 mm^3 en el grupo tratado con HNF y 270.0 ± 35.86 mm^3 en el grupo control (t-test, P<0.03). La reducción del volumen de infarto global fue de un 46% en las ratas tratadas con HNF. Esta reducción se observó tanto en el infarto cortical (51.8%; t-test, P=0.03) como en el estriado (25.8%; t-test, P=0.02). Evaluamos si el mismo efecto se mantenía a los 7 días de la reperfusión en otro grupo de 14 ratas, 7 tratadas con HNF y 7 con suero salino. En este segundo grupo tampoco se observaron hemorragias cerebrales o sistémicas. De nuevo, el tratamiento con HNF redujo el volumen de infarto (97.3 ± 20.95 mm^3 contra 242.1 ± 62.72 mm^3; t-test, P<0.03). El efecto protector de la HNF fue más marcado en la región cortical que en la subcortical (ANOVA de dos vías, P<0,05).

En el grupo de ratas en el que se midieron los valores de flujo sanguíneo cerebral durante la isquemia no se observaron diferencias entre las ratas tratadas con HNF y salino, lo que indica que el efecto protector de la HNF no se debe a un aumento del flujo sanguíneo postisquémico.

En otro experimento se evaluó el efecto de la HNF administrada a la primera hora del inicio de la isquemia. Para la realización de este experimento se utilizaron 14 ratas, 7 tratadas con HNF y 7 tratadas con salino. El protocolo fue idéntico al del grupo anterior salvo el momento de inicio del tratamiento que fue tras 1 hora de isquemia, o lo que es lo mismo, al inicio de la reperfusión. El volumen de infarto en las ratas placebo fue de 290.1 ± 41.95 mm^3, mientras que el de las ratas tratadas con HNF fue de 134.1 ± 52.49 mm^3. La

diferencia entre ambos grupos fue estadísticamente significativa: t-test; P=0.038 (figura 4).

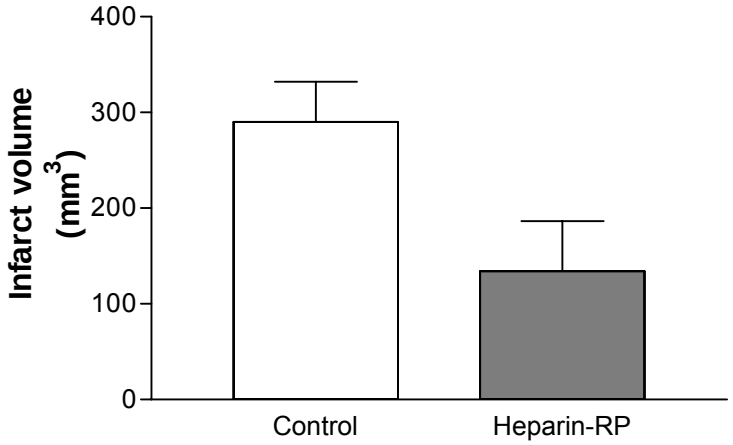

Fig. 4. Diferencias en el volumen de infarto cerebral entre las ratas tratadas con HNF o salino al inicio de la reperfusión.

Comparando el grupo tratado en la primera hora y el grupo tratado a las 3 horas, se observó que la reducción del volumen del infarto tiende a ser mayor cuando más pronto se instaura el tratamiento, aunque esta diferencia no es estadísticamente significativa (figura 5).

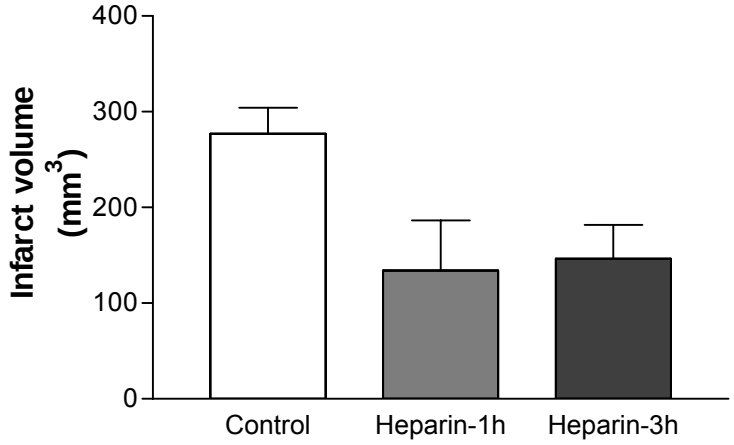

Fig. 5. Comparación entre los volúmenes de infarto entre las ratas tratadas con salino, con HNF tras 1 hora de isquemia y con HNF tras 3 horas de isquemia.

3. Efecto antiinflamatorio de la HNF en el modelo de isquemia-reperfusión cerebral focal de rata.

Los niveles plasmáticos de IL-10 fueron significativamente mayores en el grupo tratado con HNF (t-test, P=0.005). La expresión de VCAM-1 en el hemisferio isquémico se redujo un 27.4% en las ratas tratadas con HNF (test de comparación múltiple post hoc de Bonferroni, P<0.05). La expresión de HO-1 inducida por la isquemia cerebral fue significativamente mayor en el hemisferio isquémico de las ratas tratadas con HNF comparadas con el grupo control (t-test, P=0.04).

Con las muestras de plasma obtenidas de todas las ratas intervenidas se analizaron los niveles de TNF-α mediante técnicas de enzimoinmunoanálisis (ELISA). De esta manera obtuvimos los niveles plasmáticos de TNF-α a las 24 horas del inicio de la isquemia. Estos niveles fueron menores en las ratas tratadas con HNF que en el grupo placebo, aunque esta diferencia no fue significativamente diferente (figura 6).

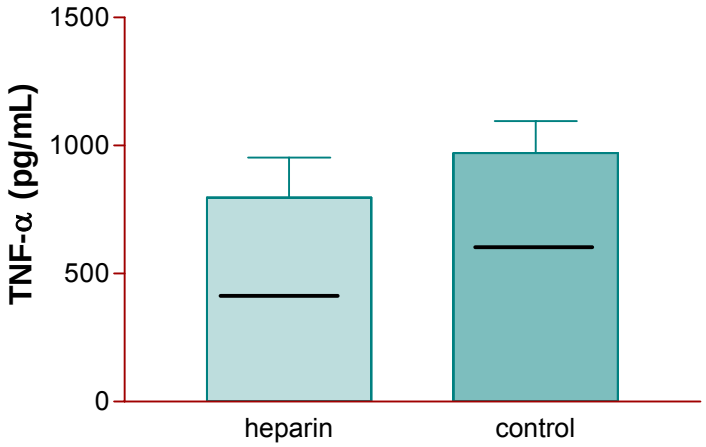

Fig. 6. Comparación de los niveles de TNF-β séricos a las 24 horas de isquemia entre las ratas tratadas con HNF y salino.

4. Marcadores séricos de inflamación en pacientes con ictus isquémicos tratados con HNF.

En el primer estudio se incluyeron 706 pacientes con ictus isquémico agudo, 450 recibieron tratamiento con dosis alta de HNF y 206 recibieron 300 mg/día de aspirina. Los pacientes tratados con HNF presentaban una mayor prevalencia de fibrilación auricular, pero no hubo otras diferencias significativas en los datos epidemiológicos o factores de riesgo vascular. El retraso en el inicio del tratamiento y la gravedad de la alteración neurológica fue similar en ambos grupos.

El número total de leucocitos, el porcentaje de polimorfonucleares y la velocidad de sedimentación globular (VSG) fue significativamente mayor en los pacientes que recibieron aspirina que en los que recibieron HNF. Aunque estas diferencias se observaron en todo el grupo como conjunto, análisis posteriores demostraron diferencias tan sólo en los pacientes con ictus no lacunares. El análisis mediante regresión linear simple mostró que el número total de leucocitos, el porcentaje de polimorfonucleares y la VSG se correlacionaban con una mayor afectación neurológica inicial (P=0.0001). La reacción de fase aguda fue significativamente menor en los pacientes con recuperación completa que en los que presentaban una recuperación incompleta. Estas diferencias sólo tuvieron significación estadística en el grupo de pacientes con un infarto cerebral no lacunar.

En modelos de ANCOVA separados se evaluó el grado de recuperación neurológica al alta en los pacientes con infarto cerebral lacunar o no lacunar. Las variables que predecían de forma independiente el pronóstico fueron el grado de afectación neurológica inicial, el ictus recurrente y el sangrado grave. Ambos modelos demostraron una asociación positiva entre el uso de HNF y la recuperación completa. Este efecto era de mayor envergadura en el grupo de pacientes con ictus no lacunar. En este grupo se encontró una asociación negativa para el número de leucocitos totales. En ninguno de estos modelos, el porcentaje de polimorfonucleares o las VSG persistieron asociados a la recuperación del ictus de forma significativa.

En el segundo estudio se incluyeron 167 pacientes consecutivos con ictus isquémicos, 70 recibieron dosis altas de HNF y 97 recibieron tratamiento con 300 mg al día de aspirina durante las primeras 24 horas desde el inicio de los síntomas. No hubieron diferencias en

la gravedad inicial del ictus en ambos grupos. Las variables que se asociaron con un mejor pronóstico (índice de Barthel>85) era la gravedad inicial (evaluada mediante la escala canadiense, la disminución de un punto OR 1.66; 1.31-2.11, P< 0.001), la anormalidad en el TC craneal inicial (OR 11:30; 3.99-32.46; P<0.001) y el aumento de temperatura axilar (por el incremento de 1 grado, OR 3.50; 1.72-7.15; P<0.001). Los valores medios de TNF-α e ICAM-1 sérica a las 48 horas fueron significativamente menores que en la determinación basal (P<0.001). Por el contrario, los valores medios de IL-6, IL-4 y VCAM-1 sérica fueron significativamente mayores en el seguimiento (P<0.01). La IL-10 fue la única citocina que no experimentó cambios significativos longitudinales. Los niveles basales de IL-6, TNF-α e ICAM-1 sérica eran mayores en los pacientes que presentaban signos precoces en el TC craneal. La VCAM-1 sérica a las 48 horas fue el único marcador inflamatorio asociado independientemente con el índice de Barthel medido a los 6 meses. Tras ajustar por los valores inflamatorios basales, la valoración neurológica inicial, los hallazgos del TC craneal inicial y la presión arterial media, los valores de VCAM-1 sérica fueron 1.24 veces mayores en el grupo tratado con aspirina que en el de pacientes tratados con HNF (1.04-1.49, P=0.017). El curso en el tiempo de los parámetros biológicos restantes no difirió entre los dos grupos de tratamiento.

5. Estudio de la eficacia clínica de la HNF en un ensayo clínico aleatorizado.

Después de 30 meses de estudio, con 67 pacientes incluidos, el reclutamiento tuvo que cerrarse. Se trataron 35 pacientes con aspirina y 32 con HNF. Los dos grupos eran similares, aunque un porcentaje mayor de pacientes tratados con HNF presentaban infartos extensos en el TC craneal inicial. La única variable pronóstica diferente entre ambos grupos fue una menor tendencia a la recurrencia isquémica en pacientes tratados con HNF. No se encontraron diferencias en los valores de presión arterial, temperatura o glucemia. Los niveles deseados de heparinemia se alcanzaron tan sólo en el 41 % de pacientes en las primeras 24 horas. El empeoramiento del ictus isquémico se asoció con niveles de heparinemia más bajos que en los casos de ictus estables. El empeoramiento por causas hemorrágicas se asoció con niveles altos de heparinemia. La HNF demostró un perfil de seguridad igual al de la aspirina.

V. DISCUSIÓN.

En el estudio presentado en el primer trabajo encontramos la pauta de administración de HNF en rata que mantenía una heparinemia entre 0.3 y 0.5 U/mL, que es el objetivo marcado. Esto nos permitió adaptar nuestro modelo animal de isquemia para conseguir utilizar el tratamiento anticoagulante de una manera similar a la que se utiliza en la práctica clínica diaria. Dada la falta de monitorización adecuada de la farmacodinamia de la HNF en los ensayos clínicos realizados en pacientes con ictus isquémico y la evidencia en estudios previos de que la seguridad del fármaco depende, en gran medida, de su monitorización estrecha (Chamorro et al, 1999), este punto fue fundamental en el desarrollo de nuestros experimentos.

Tal y como demostraron Yanaka y colaboradores (1996), la HNF es neuroprotectora en el modelo de isquemia cerebral focal de rata. Así, este hecho se ha corroborado en, al menos, dos laboratorios diferentes, lo que constituye uno de los requisitos del grupo STAIR para el estudio de fármacos neuroprotectores (Fisher et al, 2005).

Un nuevo hallazgo de este estudio es que la administración de HNF se asoció con niveles plasmáticos elevados de IL-10, una molécula antiinflamatoria principalmente secretada por linfocitos y monocitos/macrófagos que puede suprimir la producción de moléculas proinflamatorias como TNF-α, IL-1 e IL-8 (Tedgui and Mallat, 2001). Los efectos beneficiosos de la HNF también incluyeron una menor expresión de VCAM-1 endotelial, que podría favorecer el daño cerebral al inducir la expresión de factor tisular (McGilvray et al., 1997). La administración de HNF también se asoció con un aumento de la expresión cerebral de la chaperona intracelular inducible HO-1 tras la isquemia. Esta molécula está involucrada en la supervivencia celular y la recuperación (Sharp et al., 1999). La HO-1 regula la renovación de la proteína hemo, el metabolismo del hierro y el estrés oxidativo. El aumento en la producción de HO-1 protege los vasos contra la lesión mediada por el hemo y la hemoglobina (Mazza et al, 2003), la muerte astrocitaria mediada por peróxido de hidrógeno (Fauconneau et al., 2002), y es neuroprotector en la isquemia cerebral focal (Panahian et al., 1999). Además, la inyección intramiocárdica del gen de la HO-1 en el corazón normal de la rata previo al ligamiento de una arteria coronaria causa una importante reducción en el tamaño del infarto (Melo et al, 2002). El aumento de la expresión de HO-1 que observamos en los animales tratados con HNF podría considerarse como un marcador del aumento de la supervivencia celular en el cerebro de la rata.

Así, en nuestro modelo experimental de isquemia cerebral focal de rata, la administración de HNF a dosis ajustada tuvo efectos neuroprotectores mediados, al menos en parte, por mecanismos antiinflamatorios.

En el segundo trabajo, en una serie de pacientes tratados con HNF o aspirina, encontramos que la liberación de reactantes de fase aguda fue menos marcada en el grupo de pacientes tratados con HNF. El número total de leucocitos, el porcentaje de polimorfonucleares y la VSG fueron menores en el grupo de pacientes anticoagulados, y esta diferencia fue estadísticamente significativa. Estos cambios se restringieron al grupo de pacientes con infarto cerebral de tipo no lacunar. Además, esta menor respuesta de fase aguda se asoció independientemente con una recuperación completa de forma precoz. A pesar de tratarse de marcadores inespecíficos de respuesta inflamatoria y de ser un estudio abierto, se trata de un acercamiento al potencial efecto antiinflamatorio de la HNF en el tratamiento de pacientes con ictus isquémico agudo.

En el tercer trabajo, en pacientes con ictus isquémico agudo, encontramos una relación inversa entre el aumento de VCAM-1 sérica y la posibilidad de una recuperación funcional, independiente de la afectación neurológica inicial y de los hallazgos en el TC craneal. Así, cuando se duplican los niveles basales de VCAM-1 sérica a las 48 horas se duplica la posibilidad de presentar un mal pronóstico. El curso temporal de IL-6, IL-4, IL-10, TNF-α e ICAM-1 sérica no se asoció con el pronóstico del ictus a los 6 meses, y la concentración de estas sustancias no difirió entre los pacientes tratados con HNF o aspirina. Contrariamente, el aumento de la VCAM-1 sérica a las 48 horas era menos pronunciado en los pacientes tratados con altas dosis de HNF que en los tratados con aspirina. Aunque no se pudo excluir que la HNF o la aspirina modularan otros marcadores inflamatorios, la VCAM-1 sérica surgió como el único marcador inflamatorio asociado con el pronóstico funcional. Creemos que estos resultados reflejan diferencias antiinflamatorias verdaderas entre la HNF y la aspirina y cuya validez está reforzada porque los pacientes se evaluaron prospectivamente.

En base a estos hallazgos, argumentamos que la anticoagulación temprana permite un modulación más eficaz del daño tisular cerebral mediado por la VCAM-1. La VCAM-1 es un factor de adhesión que se expresa tras la activación endotelial por citocinas y que se une selectivamente a células mononucleares como los linfocitos y los monocitos (von Andrian, 2001). La VCAM-1 contribuye a la adhesión firme y a la migración transendotelial

de las células mononucleares cuando sus receptores están en un estado de alta afinidad. Estos receptores son expresados por linfocitos, monocitos, eosinófilos y basófilos, pero no por los neutrófilos circulantes (von Andrian, 2001). Se ha observado una expresión intensa de VCAM-1 por los astrocitos y células endoteliales del tejido infartados, pero no de las áreas no infartadas (Blann et al, 1999). La VCAM-1 podría facilitar el daño cerebral a través de la expresión de factor tisular, ya que la unión de las integrinas monocitarias con la VCAM-1 endotelial induce la expresión de factor tisular (McGilvray et al, 1997). El factor tisular se expresa difusamente en astrocitos y células aracnoideas meníngeas (Eddleston et al, 1993) y representa el iniciador celular primario de la cascada de la coagulación extrínseca (Bach, 1988). La HNF elimina el aumento de monocitos positivos para factor tisular inducido por la endotoxina in vivo (Pernerstorfer et al, 1999) e incrementa los niveles plasmáticos del inhibidor de la vía del factor tisular (Sandset et al, 1988). El efecto inhibidor de la HNF sobre el factor tisular está mediado por su habilidad para reducir la expresión de IL-1β, TNF-α, IL-6 y las L- y P-selectinas, que a su vez aumentan la producción de factor tisular (Gori et al, 1999). Sin embargo, los mecanismos moleculares por los que la HNF disminuye los niveles de VCAM-1 sérica en el ictus isquémico agudo son desconocidos.

El último trabajo, un ensayo clínico aleatorizado comparando la eficacia de la HNF y la aspirina en pacientes con ictus isquémico agudo no lacunar, fue interrumpido prematuramente por falta de financiación. Dada la complejidad del estudio y el ámbito multicéntrico internacional, la dosis utilizada de HNF tuvo que ser más baja de lo previsto, por lo que a las 24 horas del inicio del tratamiento tan sólo un 41% de los pacientes tratados con HNF alcanzaron unos niveles terapéuticos óptimos. Los niveles subóptimos de HNF se asociaron con empeoramiento isquémico del ictus, mientras que la transformación hemorrágica se asoció con niveles elevados de HNF. A diferencia de los estudios previos con dosis fijas y no monitorizadas de HNF (International Stroke Trial Collaborative Group, 1997), en el estudio RAPID la HNF era al menos tan segura como la aspirina, aunque el estudio no puede excluir un teórico aumento del empeoramiento hemorrágico 9 veces mayor. Sin embargo, el estudio RAPID sugiere que el riesgo hemorrágico puede disminuirse mediante una monitorización estrecha y un ajuste del TTPA. El empeoramiento del ictus isquémico se asoció con una heparinemia menor de 0.3 U/mL, que se encontró en más de la mitad de pacientes durante el primer día de tratamiento. Así, se podría haber conseguido un mayor beneficio si los valores de heparinemia deseados se hubieran alcanzado más rápidamente. El estudio RAPID no

tiene la potencia adecuada, pero es el mayor ensayo clínico comparando el riesgo-beneficio de la aspirina y la HNF a dosis alta ajustada al peso en el ictus isquémico agudo. En base a nuestros hallazgos, la ausencia de evidencia no debería ser equiparada a una evidencia de efectos negativos. Contrariamente, nosotros defendemos la realización de otro ensayo clínico que evalúe en 1.184 pacientes con ictus isquémico agudo si la administración temprana de la HNF ajustada al peso es neuroprotectora, con una diferencia del 8% respecto al tratamiento con aspirina. Mientras tanto el papel de la HNF en el ictus isquémico agudo es un asunto abierto.

Por otra parte, se ha publicado recientemente un ensayo aleatorizado controlado en el que se administraba HNF o placebo durante 5 días en los ictus de menos de 3 horas de duración (Camerlingo et al, 2005). El objetivo primario del estudio, que era encontrar diferencias en muerte o dependencia a los 3 meses, se cumplió, ya que los pacientes con buena recuperación en el grupo de HNF fueron un 39%, mientras que en el grupo placebo fueron 29% (P=0.025). Así, la HNF administrada en las 3 primeras horas del inicio de los síntomas fue beneficiosa en el infarto cerebral hemisférico no lacunar, con un disminución del riesgo absoluto de recuperación o independencia del 10%. Las hemorragias fueron superiores en el grupo de pacientes tratados con HNF, pero el efecto clínico global era beneficioso.

La lectura conjunta de los 4 trabajos que presentamos permite extraer la conclusión de que la HNF tiene un papel neuroprotector. En nuestro modelo de isquemia cerebral focal de rata este papel neuroprotector está mediado por efectos antiinflamatorios. Estos mismos efectos han sido demostrados en series clínicas de pacientes tratados con HNF. Sin embargo, este efecto no ha podido confirmarse en un ensayo clínico aleatorizado, dada la complejidad de este tipo de estudios en el ictus isquémico agudo, sobre todo cuando no existe el soporte económico adecuado. Así, tal como se demuestra en las controversias en el ictus (Controversies in Stroke) del último número publicado de Stroke, la revista internacional líder en la clínica vascular cerebral, el tratamiento anticoagulante precoz en la isquemia cerebral sigue siendo un tema abierto (Chamorro, 2006; Sandercock, 2006; Davis y Donnan, 2006). Sería deseable resolver esta cuestión en el futuro mediante un estudio RAPID II adecuadamente financiado.

VI. CONCLUSIONES

1. Una dosis de heparina no fraccionada de 70 U/kg/h precedida de un bolus de 200 U/kg permite mantener una heparinemia constante entre 0.3 y 0.6 U/mL en la rata. Los niveles de heparinemia se alcanzan rápidamente y se mantienen de forma constante en el tiempo. El tiempo parcial de tromboplastina activado se correlaciona muy bien con los niveles de heparinemia.

2. La heparina no fraccionada es neuroprotectora en el modelo de isquemia-reperfusión cerebral focal de rata. Este efecto se manifiesta con una reducción del volumen de infarto del 46% cuando el fármaco es administrado 3 horas después del inicio de la isquemia. La reducción del infarto se produce tanto a nivel cortical como subcortical y se mantiene hasta el día 7 después de la isquemia.

3. La acción neuroprotectora de la heparina no fraccionada está mediada, en parte, por mecanismos antiinflamatorios. En las ratas tratadas con heparina no fraccionada se evidencia un incremento los niveles plasmáticos de la interleucina-10, que es antiinflamatoria. Asimismo, en el tejido cerebral existe un aumento de la expresión de la hemooxigenasa-1. Por otra parte hay una inhibición de la inducción de la molécula de adhesión vascular celular-1 en el endotelio cerebral isquémico, lo que es un mecanismo antiinflamatorio.

4. En los pacientes con ictus isquémico agudo tratado con heparina no fraccionada se observa una disminución de ciertos marcadores inflamatorios en comparación con los pacientes tratados con aspirina. Los pacientes tratados con heparina no fraccionada presentaban un menor número total de leucocitos, porcentaje de polimorfonucleares y velocidad de sedimentación globular. Así como unos niveles séricos más bajos de la molécula de adhesión vascular celular-1. Esta disminución de marcadores inflamatorios se asoció con una mejor recuperación funcional tras el ictus.

5. La medicina basada en la evidencia no tiene, en estos momentos, datos suficientes para evaluar la eficacia y seguridad del tratamiento con heparina no fraccionada en las primeras 12 horas tras un ictus isquémico no lacunar. No obstante, el estudio RAPID aporta que el perfil de seguridad de la heparina no fraccionada puede ser similar al de la aspirina si el fármaco es debidamente monitorizado y ajustado, y una mayor eficacia en la prevención de recurrencias tempranas.

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