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AVALIAÇÃO DOS PRODUTOS DA DEGRADAÇÃO DA HEMOGLOBINA NA LESÃO CEREBRAL E MECANISMOS DE PROTEÇÃO ENCEFÁLICA APÓS A HEMORRAGIA INTRACRANIANA

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"In seeking wisdom, thou art wise; in imagining that thou hast attained it – thou art a fool ".

Lord Chesterfield

RESUMO

Introdução: O acidente vascular encefálico hemorrágico e a hemorragia subaracnóide são doenças de elevada morbi-mortalidade. Os produtos da degradação da hemoglobina são implicados em diversos estudos experimentais como elementos-chave na fisiopatologia da lesão secundária após a hemorragia intracraniana. Entretanto, há poucos dados em humanos que possam corroborar as observações experimentais. Objetivo: Avaliar o papel dos produtos da degradação da hemoglobina e dos mecanismos de proteção contra a hemoglobina e o heme na fisiopatologia do dano secundário à hemorragia intracraniana. Métodos: Estudo prospectivo realizado nas unidades neurointensivas de três hospitais. Foi coletado sangue e líquor (pela DVE) de pacientes internados com AVEh ou HSA e hemoventrículo durante os primeiros três dias após o ictus. Foram dosadas sequencialmente as concentrações de ferro, heme, hemopexina, haptoglobina, enolase e S100-β além de um painel de citocinas. O desfecho primário era mortalidade em 7 dias. Resultados: Quinze pacientes foram incluídos, 10 com HSA e 5 com AVEh. Após a hemorragia intracraniana, ocorreu o desencadeamento da resposta inflamatória no sistema nervoso central (SNC), com níveis de IL-8 e GM-CSF no líquor cerca de 20x superiores ao do plasma. Foi observada a correlação entre a concentração de ferro e IP-10 no líquor ($r=0,97$; $p=0,03$) e heme e MIP-1b no líquor ($r=0,76$; $p=0,01$). Os níveis de hemopexina e haptoglobina foram consistentemente inferiores no líquor em relação ao plasma, ao longo dos três dias de estudo. Tanto o ferro e heme plasmáticos, quanto o grau de resposta inflamatória sistêmica e no SNC foram preditores de mortalidade nos primeiros 7 dias após o evento. Conclusão: Os resultados desse estudo mostram que tanto o ferro quanto o heme estão correlacionados ao desencadeamento da lesão secundária após a hemorragia intracraniana e estão associados ao pior prognóstico neste grupo de pacientes. Além disso, os mecanismos de proteção cerebral contra a hemoglobina e o heme são insuficientes. Mais estudos são necessários para elucidar o papel dos produtos da degradação da hemoglobina na fisiopatologia da hemorragia intracraniana em humanos.

ABSTRACT

Introduction: Hemorrhagic stroke and subarachnoid hemorrhage are diseases with high morbidity and mortality. Hemoglobin degradation byproducts are being increasingly implicated in the pathophysiology of secondary brain injury after intracranial bleeding. However, there is not enough data in humans to support experimental evidence. Objective: To evaluate the role of hemoglobin degradation byproducts and protective mechanisms against hemoglobin and heme in the pathophysiology of secondary brain injury. Methods: Prospective study was done in three neurocritical care units. Blood and cerebrospinal fluid from EVD were collected from hemorrhagic stroke and subarachnoid hemorrhage patients throughout the first three days after the ictus. Sequentially measurement of iron, heme, haptoglobine, hemopexine, enolase, s100-β and cytokines were performed. Primary outcome was 7-day mortality. Results: Fifteen patients were included, 10 with subarachnoid hemorrhage and 5 with hemorrhagic stroke. After intracranial bleeding, local inflammatory response was elicited, with CSF IL-8 and GM-CSF levels 20x higher than in plasma. There is a correlation between CSF iron and IP-10 levels ($r=0.97$; $p=0.03$) and between CSF heme and MIP-1b concentration ($r=0.76$; $p=0.01$). Throughout the first three days after the event, CSF hemopexine and haptoglobine concentrations were consistently lower than in plasma. Both CSF iron and heme levels and systemic and local inflammatory response were predictors of early mortality. Conclusion: The results of this study demonstrate that iron and heme are related to secondary brain injury after intracranial bleeding and are predictors of poorer prognosis. Moreover, mechanisms of protection against hemoglobin and heme are lacking. More studies are needed to clarify the role of hemoglobin metabolism byproducts in the pathophysiology of intracranial bleeding in humans.

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LISTA DE ABREVIATURAS

AVE – acidente vascular encefálico

AVEi – acidente vascular encefálico isquêmico

AVEh – acidente vascular encefálico hemorrágico

BHE – Barreira Hematoencefálica

CO – monóxido de carbono

DVE – derivação ventricular externa

ELISA - *Enzyme Linked Immuno Sorbent Assay*

GM-CSF – *Granulocyte-macrophage colony stimulating factor*

HMGB-1 – *High mobility group box* -1

HO – Heme-oxigenase

Hp – Haptoglobina

HRP – *horseradish peroxidase*

HSA – hemorragia subaracnóide

Hx - Hemopexina

ICAM-1 – *Intercellular adhesion molecule* -1

IL - Interleucina

IL-1β – Interleucina -1β

IP-10 – *Interferon gamma-induced protein* 10

MCP-1 - proteína quimiotática para monócitos-1

MIP-1b - *macrophage inflammatory protein* 1b

MMP-9 – metaloproteinase de matriz 9

MPO - mieloperoxidase

NF-κβ - *nuclear factor kappa-light-chain-enhancer of activated B cells*

NMDA - N-Metil D-Aspartato

Nrf2 – *NF-E2-related factor* 2

NSE- *Neuron-specific enolase*

RNAm – ácido ribonucleico mensageiro

Rt-PA- Ativador do plasminogênio tecidual recombinado

SARA – síndrome da angústia respiratória aguda

SNC – Sistema nervoso central

SOD – superóxido dismutase

SRIS – Síndrome da Resposta Inflamatória Sistêmica

TLR – *Toll-like receptor*

TMB - 3,3',5,5'-tetrametilbenzidina

TNF-α – Fator de necrose tumoral α

Treg – T reguladores

VCAM-1 – *Vascular cell adhesion protein*-1

1. INTRODUÇÃO

O acidente vascular encefálico (AVE) é classicamente caracterizado como um déficit neurológico atribuível a uma lesão focal aguda do sistema nervoso central (SNC) de etiologia vascular (Sacco et al., 2013). A definição de AVE inclui o infarto cerebral, a hemorragia intracerebral e a hemorragia subaracnóide (HSA) e essas entidades são uma grande causa de morbi-mortalidade mundialmente. Anualmente, 15 milhões de pessoas sofrem um episódio de AVE no mundo inteiro. Dessas, cinco milhões morrem e outras cinco milhões sofrem sequelas permanentes (Mackay et al, 2004). Nos países desenvolvidos, a doença cerebrovascular é a terceira maior causa de morte, sendo superada apenas pelas doenças cardiovasculares e pelas neoplasias. No Brasil, e também no estado do Rio de Janeiro, o AVE é a primeira causa de óbito ("Informações de Saúde: Estatísticas Vitais. Disponível Em: Http://tabnet.datasus.gov.br." 2013).

A hemorragia intracraniana é definida como hemorragia no interior da caixa craniana e os seus subtipos são definidos de acordo com o sítio do sangramento. A hemorragia intracerebral é caracterizada por uma coleção focal de sangue no interior do parênquima cerebral ou do sistema ventricular que não é causada por trauma, enquanto a HSA é definida pelo sangramento no espaço subaracnóide (Sacco et al., 2013). Já o AVE hemorrágico (AVEh) é definido como a presença de sinais clínicos rapidamente progressivos de disfunção neurológica atribuída à hemorragia intracerebral.

Do total de episódios de AVE, cerca de 87% são isquêmicos, 10% hemorrágicos e, 3% são de hemorragia subaracnóide (World Health Organization, 2006). Estes números se traduzem em cerca de 103000 casos de AVEh por ano nos Estados Unidos e 2 milhões de casos mundialmente (van Asch et al., 2010). A hemorragia intracraniana é uma entidade de elevada morbimortalidade, com a letalidade variando entre 27-44%. A presença de hemoventrículo aumenta a letalidade para 50-75% dos casos (Hemphill et al., 2001). Essas

elevadas taxas de morbi-mortalidade provavelmente resultam do fato que não há opções efetivas de tratamento para melhorar a sobrevida do paciente após a hemorragia intracraniana. O tratamento padrão é limitado a terapias de suporte como controle da pressão intracraniana, tratamento do edema cerebral e manutenção da estabilidade ventilatória e hemodinâmica (Flower et al, 2011). Por isso, o entendimento dos mecanismos fisiopatológicos é de fundamental importância para identificação de novos alvos terapêuticos e para o desenvolvimento de novas terapias contra a hemorragia intracraniana. Neste trabalho, iremos nos referir à hemorragia intracraniana como hemorragia no interior da caixa craniana e ao AVE hemorrágico como sangramento no interior do parênquima encefálico ou nos ventrículos.

1.1 - EPIDEMIOLOGIA

A epidemiologia do AVEh varia bastante com a população estudada e, de forma geral, tem se mantido constante ao longo dos anos. Uma meta-análise que incluiu 36 estudos realizados entre 1980-2008 e 8145 pacientes com AVEh mostrou que a incidência é de 24.6/100.000 pessoas-ano e que vem se mantendo estável durante os períodos das coortes. A incidência também aumenta com a idade (aumento maior que 10 vezes na faixa etária > 85 anos quando comparada a de 45-54 anos) e é maior na população asiática (51.8/100.000 pessoas-ano)(van Asch et al., 2010). Interessantemente, estudo francês que avaliou a incidência de AVEh em Dijon de 1985 a 2008 mostrou que, apesar da incidência geral ter se mantido constante ao longo dos anos (em torno de 12,4/100.000 pessoas-ano), a incidência na faixa etária ≥ 75 anos aumentou 80%, o que contrasta com a redução de 50% da incidência na faixa etária < 60 anos (Béjot et al., 2013). Os autores especulam que a redução da incidência na faixa etária mais jovem pode estar relacionada ao melhor controle de fatores de risco

enquanto o aumento nos idosos pode estar relacionado à maior utilização de antiplaquetários e anticoagulantes.

Em relação à HSA, também existe uma variação considerável na sua incidência anual ao redor do mundo. Um estudo patrocinado pela Organização Mundial da Saúde mostrou variabilidade na incidência de até 10 vezes entre países asiáticos e europeus, indo de 2,0 casos/100.000 pessoas-ano na China até 22,5 casos/100.000 pessoas-ano na Finlândia (Ingall et al., 2000). Revisão sistemática realizada posteriormente sugere que a incidência ajustada pela idade é o dobro nos países em desenvolvimento quando comparado aos países desenvolvidos (Feigin et al., 2009). A estimativa da incidência nos Estados Unidos é de 14,5 casos/100.000 pessoas-ano (Shea et al., 2007). Entretanto, considerando que cerca de 12-15% dos pacientes com HSA morrem antes de chegar ao hospital, a real incidência deve ser maior (Schievink et al., 1995). A incidência de HSA também sofre variação de acordo com a idade, gênero e etnia – ela aumenta com a idade, principalmente acima dos 50 anos, é maior em mulheres que em homens e mais comum em negros e hispânicos que em caucasianos (10),(Labovitz et al., 2006).

Existem poucos estudos que avaliaram a incidência da hemorragia intracraniana no Brasil. Minelli et cols mostraram que a incidência de AVE no município de Matão, São Paulo, era de 108 casos/100.000 habitantes, sendo que 13,6% dos casos eram de AVEh (Minelli et al, 2007). Outro estudo realizado em Joinville, Santa Catarina, mostrava incidência de 9,5 casos/100.000 pessoas-ano para o AVEh e de 5,6 casos/100.000 pessoas-ano para a HSA (Cabral et al., 2009). Mais recentemente, estudo realizado em Fortaleza, Ceará mostrou que, dos 2418 pacientes admitidos com diagnóstico de AVE, 15,2% eram portadores de AVEh e 6%, de HSA (Carvalho et al., 2011).

A mortalidade do AVEh foi estimada em cerca de 40% no primeiro mês após o evento (sendo menor no Japão que no resto do mundo, ao redor de 16,7%) e tem se mantido

relativamente estável ao longo dos últimos 20 anos (van Asch et al., 2010). Apenas 12-39% dos sobreviventes são capazes de realizar as atividades do cotidiano (van Asch et al., 2010). Estudo epidemiológico italiano mostrou que a taxa de mortalidade precoce também foi bastante elevada, sendo de cerca de 35% na primeira semana após o evento, chegando a 50,3% em um mês e 59% em um ano (Sacco et al., 2009). Dos pacientes que morrem na primeira semana, 98,4% o fazem por causa neurológica (Sacco et al., 2009). A mortalidade a longo prazo também parece ser mais elevada que na população geral – estudo sueco que acompanhou 323 pacientes portadores de AVEh por 13 anos mostrou que a sobrevida ao final deste período foi de 34%, comparada a 61% da população geral, e que as causas de morte mais comuns eram AVE recorrente e doença isquêmica cardíaca (Hansen et al., 2013).

A mortalidade atribuída à HSA permanece alta em todo o mundo. Meta-análise recente que incluiu 33 estudos e 8739 pacientes mostrou que a mediana da taxa de mortalidade dos estudos publicados nos Estados Unidos foi de 32% comparado a 43-44% na Europa e 27% no Japão (Nieuwkamp et al., 2009). Entretanto, a taxa de mortalidade vem diminuindo ao longo do tempo – cerca de 0,6% ao ano, perfazendo uma redução de cerca de 17% ao longo de três décadas. Esse números vem de estudos que não levam em consideração os casos de óbito pré-hospitalar. Esta é uma consideração importante, porque a redução da mortalidade tem se dado às custas do aumento da sobrevida dos pacientes hospitalizados após um episódio de HSA. A mortalidade também parece ser influenciada pelo gênero e pela etnia – mulheres apresentam maior mortalidade que homens (Johnston et al, 1998) e negros e indígenas maior que caucasianos (Ayala et al., 2001).

Já no Brasil, a mortalidade estimada nos estudos populacionais varia entre 41% e 45,4% em 30 dias e 49,4% em 6 meses para o AVEh (Minelli et al, 2007), (Cabral et al., 2009). Em relação à HSA, a mortalidade encontra-se entre 36,4% em 30 dias e 49,4% em 6 meses (Minelli et al, 2007), (Cabral et al., 2009). Entretanto, a mortalidade da hemorragia

intracraniana vem reduzindo no Brasil nos últimos anos. De 1985 até 2009, a mortalidade por AVEh declinou em cerca de 12% para homens e de 8% para mulheres. Já no período de 1979 até 2009, a mortalidade por HSA também reduziu em cerca de 8,4% para as mulheres. Entretanto, em relação a população masculina, houve um aumento da mortalidade de 13% no período de 1979 a 2001, seguido de um pequeno decréscimo de 2,9% no período de 2002 até 2009 (Lotufo et al., 2013).

1.2 – HEMORRAGIA INTRAVENTRICULAR

A hemorragia intraventricular é definida como a presença de sangue no espaço intraventricular, geralmente secundária ao AVEh ou à HSA. Antes da era da tomografia computadorizada (TC), a presença de hemoventrículo era sugerida apenas pelas suas manifestações clínicas, como redução do nível de consciência secundária à hidrocefalia ou disfunção do tronco encefálico. Entretanto, em 1977, Little et cols publicaram a primeira série de pacientes com hemoventrículo diagnosticado pela TC de crânio (Little et al, 1977), evidenciando, a partir daí, a importância da hemorragia intraventricular como complicação frequente da hemorragia intracraniana.

A incidência do hemoventrículo varia de 30-50% dos casos de AVEh (Balami et al, 2012) e de 13 a 50% dos pacientes com HSA (Mayfrank et al., 2001), (Mohr et al., 1983). A presença de hemorragia intraventricular é um marcador independente de mau prognóstico (Claassen et al., 2001). Na ausência de tratamento específico, o hemoventrículo está associado a um risco de morte de 78% e 90% de risco de mau prognóstico (Nieuwkamp et al., 2000). Mesmo quando tratado adequadamente, a mortalidade dos pacientes com hemoventrículo varia entre 50-75% (Hemphill et al., 2001), sendo que , em um estudo, a mortalidade em 30 dias do AVEh foi de 43% naqueles com hemoventrículo contra 9% nos que não tinham hemoventrículo (Tuhrim et al., 1999). Em pacientes com HSA, a mortalidade

é de cerca de 67% naqueles com hemoventrículo, mesmo quando tratados com a derivação ventricular externa (DVE), e a chance de mau prognóstico foi de 87% (Nieuwkamp et al., 2000).

1.3 – FISIOPATOLOGIA

1.3.1 – Mecanismos de Lesão

Existem dois mecanismos principais de lesão primária decorrente da hemorragia intraventricular. O primeiro mecanismo é o de hidrocefalia aguda e o segundo mecanismo decorre do efeito de massa exercido pelo próprio coágulo intraventricular, independente da hidrocefalia aguda.. Mayfrank et cols foram os primeiros a demonstrar de forma inequívoca que a hemorragia intraventricular é o fator causal da hidrocefalia aguda como resultado da resistência exercida pelos coágulos sanguíneos sobre o fluxo liquórico (Mayfrank et al., 2001). A resistência à circulação do líquor leva ao seu acúmulo no espaço intraventricular, dilatação ventricular e aumento da pressão intracraniana, a qual, se não controlada, levará a redução do fluxo sanguíneo cerebral (Diringer et al, 1998). Em relação ao efeito de massa exercido pelo coágulo intraventricular, esse fato já foi observado por alguns autores que sugeriram que o prognóstico sombrio da hemorragia de terceiro e quarto ventrículos pode se dever, em parte, à compressão do tronco encefálico pelo coágulo no interior do ventrículo (Shapiro et al, 1994).

A lesão secundária que se segue ao hemoventrículo pode ser considerada como emanando de um progresso tempo-dependente de três processos interligados na região adjacente ao hematoma: inflamação, lise de hemácias e produção de trombina. Todos os três levam a disjunção da barreira hemato-encefálica (BHE), evoluindo direta ou indiretamente a formação de edema cerebral e morte das células do parênquima encefálico. Nesse trabalho, iremos nos concentrar nos aspectos da lesão secundária decorrentes da hemorragia

intraventricular, principalmente no dano secundário aos produtos da degradação da hemoglobina.

1.3.2. Resposta inflamatória

A inflamação é uma resposta de defesa importante do organismo secundária à hemorragia intracraniana. A resposta inflamatória começa imediatamente após a entrada dos componentes do sangue no parênquima cerebral e é caracterizada pelo acúmulo e ativação de células inflamatórias. A microglia e astrócitos residentes são os primeiros tipos celulares a responder à presença de componentes do sangue no espaço extravascular (Rolland et al., 2013), (Wang et al, 2007). A resposta inflamatória que se segue a rápida ativação da microglia envolve a infiltração de várias células inflamatórias circulantes, incluindo leucócitos, macrófagos e linfócitos T (Aronowski et al, 2005), (Gao et al., 2008). Subsequentemente, as células inflamatórias ativadas liberam um enorme gama de citocinas, quimiocinas, radicais livres e outras moléculas potencialmente lesivas (Aronowski et al, 2005), (Zhou et al., 2013).

Após a lise de hemácias, que ocorre cerca de 24 horas após o evento inicial, mais substâncias citotóxicas tais como ferro, heme e hemoglobina são liberadas, o que agrava a lesão inicial. A medida que a doença progride e a morte celular ocorre, há a liberação dos chamados *danger-associated molecular patterns* (DAMPs), que induzem a infiltração leucocitária no cérebro e agravam o estímulo inflamatório inicial (Zhou et al., 2013). Nas seções subsequentes, analisaremos cada um dos componentes que participam da resposta inflamatória.

1.3.2.1. Componente Celular

A resposta inflamatória após a hemorragia intracraniana é caracterizada por uma ativação rápida da microglia residente no SNC, seguida da infiltração de células

inflamatórias circulantes, tais como neutrófilos e macrófagos (Wang et al, 2007). O papel principal da microglia é a resolução do hematoma e a fagocitose da hemoglobina e dos produtos de sua degradação. O *down-regulation* da microglia ativada leva a retenção do hematoma e necrose celular (Wang et al., 2003). Evidência experimental indica que a ativação da microglia ocorre em cerca de 1 hora após o evento hemorrágico, enquanto a infiltração de neutrófilos começa cerca de 4-5h após o ictus (Xue et al, 2000). Tanto a trombina quanto o heme parecem ser potentes ativadores da microglia (Möller et al, 2000). O *Toll-like receptor* (TLR) 4, que é ativado pelo heme (Lin et al., 2012)(Figueiredo et al., 2007), estimula a ativação da microglia e está relacionado a pior prognóstico após o AVEh em estudos experimentais e em humanos (Rodríguez-Yáñez et al., 2012), (Sansing et al., 2011).

Embora seja necessária para a resolução do hematoma, a ativação da microglia pode exacerbar a lesão cerebral. A microglia pode causar lesão encefálica através da modulação do dano excitotóxico induzido por ATP, da indução de metaloproteinase de matriz (MMP) -9 pelos astrócitos (del Zoppo et al., 2012), da disjunção da BHE e da liberação de citocinas pró-inflamatórias, tais como TNF-α (Gregersen et al, 2000), IL-1 (Fassbender et al., 2000) e HMGB-1(Murakami et al., 2011). Paralelamente a esses eventos, a microglia também medeia a produção de espécies reativas de oxigênio (*reactive oxygen species* - ROS) e a síntese de óxido nítrico (*nitric oxide* - NO) que exercem efeitos tóxicos sobre os neurônios (Almeida et al., 2001).

Os astrócitos são células fundamentais na modulação da resposta inflamatória no SNC e na manutenção da estrutura e função cerebrais. Estas células são ativadas após a hemorragia intracraniana (Wang et al, 2008) por elementos do plasma ainda desconhecidos (Koeppen et al, 1995), uma vez que a infusão de sangue total elicita uma ativação astrocitária maior que a infusão de hemácias purificadas (Goldstein et al., 2003). A ativação astrocitária se inicia cerca

de 5 dias após a lesão inicial, sendo maior na região peri-hematoma quando comparada a periferia (Goldstein et al., 2003).

Os astrócitos também exibem uma resistência aumentada ao estresse oxidativo quando comparados aos neurônios (Lucius et al, 1996), e auxiliam na sobrevivência neuronal através da liberação de ascorbato e captação de dehidroascorbato, com consequente redução do potencial redox do meio (Swanson et al, 2004). Os astrócitos modulam a resposta inflamatória através da diminuição da expressão de iNOS e da liberação de NO, bem como da geração de ROS pela microglia (Hanisch et al, 2002). Entretanto, nem todas as respostas dos astrócitos são benéficas. Os astrócitos também apresentam ações deletérias, uma vez que essas células produzem mediadores pró-inflamatórios (TNF- α, IL-1b, IL-6 e MMP-9) através da ativação de NF-$\kappa\beta$ (Pan et al., 2011), sintetizam MMP-9 em resposta ao estresse oxidativo (Tejima et al., 2007), assim contribuindo para o edema cerebral, e interagem com os neurônios na indução da depressão alastrante, a qual pode contribuir para a lesão cerebral após o AVEh e HSA.

Os neutrófilos são o subtipo leucocitário que mais precocemente infiltra o SNC após a hemorragia intracraniana. A infiltração de neutrófilos se inicia antes de 24 horas após o evento, alcança seu pico em 2-3 dias e praticamente desaparece em 3-7 dias após o ictus, uma vez que espera-se que a apoptose desse tipo celular aconteça dentro de 2 dias após sua entrada no SNC (Gong et al, 2000), (Loftspring et al., 2009). A produção de IL-1, IL-6 e IL-8 pela microglia medeia a síntese das moléculas de adesão ICAM-1, P-selectina e E-selectina no endotélio vascular os quais, por sua vez, estimulam a atração dos neutrófilos e sua migração para o parênquima cerebral (Huang et al, 2006). O heme, que é uma molécula citotóxica e pró-oxidante liberada durante o extravasamento de sangue para o parênquima cerebral, é um potente quimioatrator para neutrófilos, estimulando a migração celular e a geração de ROS através da atividade da NADPH-oxidase (Porto et al., 2007).

O papel exato dos neutrófilos na fisiopatologia da hemorragia intracraniana ainda não é conhecido. CD18, que é um membro da família das integrinas, é expressado por leucócitos e está envolvido em uma série de doenças inflamatórias (Mazzone et al, 1995). Titova et al mostraram que camundongos *knockout* para CD18 apresentavam redução do edema cerebral e da mortalidade com consequente diminuição da expressão de nitrotirosina e mieloperoxidase (MPO) em modelo de AVEh induzido por colagenase (Titova et al., 2008). A depleção neutrofílica com um anticorpo leucocitário anti-neutrófilo reduziu a expressão de MMP-9, disjunção dos vasos sanguíneos, quebra da BHE, lesão axonal e ativação astrocítica e da microglia/macrófagos (Moxon-Emre et al, 2011). Esses estudos em animais sugerem que os neutrófilos tem um papel na lesão cerebral após a hemorragia intracraniana, entretanto, seu mecanismo de ação ainda não está totalmente elucidado.

1.3.2.2. Resposta Humoral

Citocinas são proteínas que possuem um papel primordial na sinalização entre as células e na mediação da resposta inflamatória após lesão tecidual. Muitos tipos celulares envolvidos na resposta inflamatória cerebral sintetizam e liberam citocinas pró e anti-inflamatórias, tais como a microglia (del Zoppo et al., 2012), astrócitos (Tejima et al., 2007), neutrófilos (Nguyen et al, 2007), mastócitos (Strbian et al., 2009) e até mesmo os neurônios (Wu et al., 2011). IL-1 e TNF-α são propostos como elementos fundamentais na exacerbação da lesão cerebral. TNF-α é um pequeno peptídeo de 17-kDa que é expresso por astrócitos, microglia e neurônios. Estudos mostraram que os níveis de TNF- α alcançam seu pico cerca de 4 horas após a lesão, decrescendo em 12 horas (Dalgard et al., 2012) e a concentração de IL-1b aumenta cerca de 16h após o AVEh (Wagner et al., 2006). Em um estudo em humanos que analisou o tecido cerebral post-mortem de pacientes com TCE, o TNF- α apresentou níveis aumentados de RNAm (cerca de 4x) e de sua expressão proteica (3x) em apenas 17

minutos após a lesão, enquanto os níveis de RNAm e proteína da IL-1b aumentam algumas horas após o evento e permanecem elevados por vários dias após o TCE (Frugier et al., 2010).

O TNF-α está relacionado a muitas ações deletérias. TNF- α medeia a vasoconstricção e vasoespasmo (Vecchione et al., 2009) e também está associado ao edema cerebral após a HSA (Hua et al., 2006). O bloqueio de TNF-α reduziu apoptose no hipocampo de ratos (Jiang et al., 2012) e, em um estudo em humanos, os níveis elevados de TNF- α no segundo e terceiro dias após a HSA estavam associados a pior desfecho em 3 meses (Chou et al., 2012). A inibição da IL-1b em camundongos levou ao aumento da sobrevida, melhor desfecho neurológico e menos edema cerebral e disjunção da BHE (Sozen et al., 2009). Em humanos, níveis plasmáticos elevados de IL-6 e TNF- α estavam correlacionados com edema cerebral peri-hematoma e expansão do hematoma (Castillo et al., 2002), (Silva et al., 2005).

A interleucina-6 (IL-6) é uma citocina bastante estudada no contexto da síndrome da resposta inflamatória sistêmica (SIRS). Heme (Lin et al., 2012) e TNF-α (Van den Berghe et al., 2000), através da modulação da via do NF-κβ, são estimuladores potentes da secreção de IL-6. A expressão de IL-6 parece estar localizada principalmente nos astrócitos e nas células endoteliais, e seus níveis atingem o pico em 6h após o ictus (20x superior aos níveis basais) e declinam em cerca de 7 dias (Wasserman et al, 2007). Em um estudo clínico, a expressão de RNAm e da própria IL-6 dobraram dentro de 17 minutos após a lesão e chegavam a ser 25x maior cerca de 40h após (Frugier et al., 2010). Em humanos, níveis elevados de IL-6 estão associados ao pior prognóstico após o AVEh (Castillo et al., 2002), (Silva et al., 2005) e ao vasoespasmo em pacientes com HSA (Ni et al., 2011).

Interleucina-4 (IL-4) e -10 (IL-10) parecem ter um papel neuroprotetor na lesão cerebral. IL-4 é uma citocina classicamente associada à resposta Th2 e parece estar associada a regulação inflamatória no AVE. Camundongos *knockout* para IL-4 apresentam maiores volumes de infarto cerebral, pior escore neurológico e atividade espontânea reduzida quando

comparados aos cobaias controle (Xiong et al., 2011). Já foi descrito que IL-4 estimulou o efeito neuroprotetor dos tióis e do lactato e inibiu a liberação de citocinas Th1 (Garg et al, 2009). IL-10 é uma citocina anti-inflamatória que age através da inibição da expressão de IL-1 e TNF-α. Em pacientes com AVEi, níveis plasmáticos diminuídos de IL-10 estão associados a um risco 3x aumentado de deteriorização neurológica dentro de 48h após o ictus (Vila et al., 2000). Além disso, IL-10 parece mediar a função dos linfócitos Treg. Camundongos depletados de Treg que foram injetados com IL-10 apresentavam muito menos lesão cerebral que aqueles que não receberam IL-10 (Liesz et al., 2009).

1.3.3. Hemoglobina e produtos da degradação do heme

Vários estudos já implicaram a hemoglobina (Hb), o heme e o ferro como mediadores fundamentais da lesão cerebral. A lise dos eritrócitos ocorre dentro de minutos após o extravasamento de sangue para o espaço extravascular e continua por vários dias após o ictus, liberando hemoglobina e outras substâncias tóxicas, tais como o ferro e o heme (Wu et al., 2003), (Koeppen et al, 1995). Uma vez no meio extravascular, a hemoglobina é digerida e convertida em heme e, então, em biliverdina, monóxido de carbono (CO) e ferro pelas heme-oxigenases (HO). Hemoglobina, heme e ferro são moléculas citotóxicas potentes que, através de diversos mecanismos, podem potencializar a resposta inflamatória (Figueiredo et al., 2007) e também são substâncias pró-oxidantes importantes que promovem a lesão oxidativa em proteínas, ácidos nucleicos, carboidratos e lipídeos, promovendo a disjunção da sinalização intercelular e outros efeitos tóxicos (Ryter et al, 2000).

Após a lise eritrocitária, a liberação de Hb e heme podem promover o estresse oxidativo nos tecidos expostos. Há alguma evidência para basear esta hipótese: a infusão de hemácias provoca edema e lesão neurológica dias após ao evento, o que sugere que a lise eritrocitária faça parte dos mecanismos de lesão tardia (Xi et al, 1998). Entretanto, a infusão

de hemácias lisadas resulta em edema cerebral, disjunção da BHE e lesão do DNA dentro de 24 horas, demonstrando que os produtos do metabolismo da hemoglobina possuem um efeito tóxico sobre o SNC (Xi et al., 2001), (Wu et al., 2002). Falaremos, agora, sobre o papel do ferro e do heme na lesão cerebral após a hemorragia intracraniana.

1.3.3.1. Ferro

O ferro é um elemento essencial e é utilizado numa vasta gama de reações bioquímicas no SNC, tais como o metabolismo de neurotransmissores, síntese de mielina e como parte da cadeia transportadora de elétrons. O ferro pode induzir a lesão ao SNC através de vários mecanismos. Um dos mais importantes é através do estímulo à produção de ROS, com consequente diminuição da defesa anti-oxidante. Íons férricos (Fe^{+3}) podem induzir a formação de radicais hidroxila. Sadrzadeh et cols mostraram que o ferro e a hemoglobina catalisam a produção de radicais hidroxila e peroxidação lipídica (Sadrzadeh et al., 1987), (Sadrzadeh et al, 1988). Altos níveis de proteína carbonilada foram detectadas na substância branca peri-hematoma dentro de minutos após a injeção autóloga de sangue e a produção de ROS pode persistir por até 3 dias após o ictus (Wagner et al., 2002),(Wang et al., 2003). Reduções na atividade da superóxido dismutase (SOD) e aumento da fragmentação do DNA após o AVEh também já foram descritas. Além do seu papel na lesão direta às membranas celulares, ROS pode ativar os fatores de transcrição NF-κβ (Chan et al, 2001) e a proteína ativadora 1, bem como induzir a disjunção da BHE, levando a piora do edema cerebral (Pun et al, 2009). ROS também pode levar a depressão da função mitocondrial (Sripetchwandee et al., 2013). O ferro pode ainda propagar a injúria oxidativa inibindo a função de reparação do DNA e, por conta disso, atrasando o reparo do DNA em cultura de neurônios (Li et al, 2009).

Até o momento, apenas um estudo clínico avaliou a produção de ROS como mediador da lesão cerebral após o AVEh. Mantle et cols descreveram a presença de proteínas oxidadas

nas amostras de tecido cerebral peri-hematoma obtidos após a drenagem do hematoma em 10 pacientes (Mantle et al., 2001). Entretanto, evidência da produção de ROS também foi encontrada no grupo controle (pacientes submetidos a ressecção de tumores cerebrais ou clipagem de aneurisma). Supôs-se que os pacientes controle também estavam sujeitos a níveis elevados de estresse oxidativo devido a sua doença de base (tumores cerebrais e aneurismas intracranianos). A despeito da ausência de evidência clínica, dados experimentais abundantes mostram que a produção de ROS é um componente crítico na lesão cerebral após o AVEh.

Outro mecanismo de lesão cerebral pelo ferro é através da amplificação da resposta inflamatória. A micróglia ativada por lipopolissacarídeo (LPS), quando repleta de ferro, apresenta aumento da liberação de MMP-9 (Mairuae et al, 2011), TNF- α e IL-1b comparada a microglia depletada de ferro (Zhang et al., 2006). O meio de cultura da microglia ativada se mostrou tóxico para oligodendrócitos; este efeito era revertido com quelantes de ferro. Além disso, aumento dos níveis de ferro também levaram à ativação de NF-$\kappa\beta$ (Zhang et al., 2006).

A excitotoxicidade pelo glutamato parece ser um mecanismo importante de morte neuronal e dos oligodendrócitos. O glutamato promove a captação de ferro em explantes e medula espinhal de ratos (Yu et al., 2009) e, por outro lado, o ferro medeia os efeitos tóxicos do glutamato e estimula a liberação do mesmo através do aumento da atividade da aconitase (Schalinske et al, 1998), que é uma enzima importante para a síntese do glutamato. O glutamato também parece aumentar a permeabilidade da BHE e aumentar o edema cerebral através dos receptores NMDA, que são estimulados pelo estresse oxidativo e inibidos por quelantes de ferro (Germanò et al., 2007), (Liu et al., 2010), (Im et al., 2012). Além disso, um corpo de evidência crescente sugere que o ferro pode induzir a neurodegeneração, promover a autofagia neuronal (Chen et al., 2012), aumentar a neurotoxicidade da proteína β-amilóide através da expressão da transglutaminase (Wang et al., 2012) e causar atrofia e morte neuronal (Caliaperumal et al, 2012). Em humanos, a ferritina sérica (Mehdiratta et al., 2008) e

o conteúdo de ferro do hematoma (Lou et al, 2009) (mensurado através de ressonância nuclear magnética) estão relacionados à progressão do edema cerebral.

1.3.3.2. Hemoglobina e Heme

A Hb livre é um elemento oxidativo que reage com o óxido nítrico (NO) e com outros oxidantes fisiológicos, tais como o peróxido de hidrogênio e o peróxido de lipídeos. A depleção de NO induzida pela Hb leva a geração de nitrato e a formação do íon férrico ligado a Hb (Fe^{+3}), que acumula dentro dos tecidos e promove a reação do heme com proteínas e lipídeos. O consumo de NO também parece mediar a vasoconstricção na HSA (Sabri et al., 2011) e pode explicar a resposta hipertensiva aguda comumente vista na hemólise maciça.

A oxidação da Hb pode levar a formação de heme (Olson et al., 2004), (Minneci et al., 2005). Heme pode oxidar proteínas e lipídeos, principalmente LDL (Jeney et al., 2002) e promover lesão tecidual (Nagy et al., 2010). Modelos in vitro mostraram que neurônios e astrócitos são sensíveis aos efeitos tóxicos do heme livre e, aparentemente, seus efeitos nocivos também são mediados através de mecanismos independentes do ferro (Chen-Roetling et al, 2006).

Além de estimular diretamente o estresse oxidativo, o heme também pode participar da resposta inflamatória através do estímulo direto do TLR-4 (Ryter et al, 2000). O heme também pode induzir a migração e ativação dos neutrófilos (Graça-Souza et al., 2002), geração de ROS (Porto et al., 2007), e a produção de TNF- α (Fortes et al., 2012) e IL-8 (Lou et al, 2009). Além disso, o heme parece induzir a expressão de moléculas de adesão pró-inflamatórias tanto in vitro (Wagener et al., 1997) quanto in vivo (Wagener et al., 2001), além de promover o aumento da permeabilidade vascular (Graça-Souza et al., 2002, -), perpetuando o edema cerebral. Além de promover a resposta inflamatória no SNC, o heme também parece induzir a necrose celular em macrófagos in vivo (Mantle et al., 2001). Finalmente, os neurônios são mais sensíveis aos efeitos tóxicos do heme (Lara et al., 2009) e da hemoglobina

(Chen-Roetling et al, 2006) comparados aos astrócitos, o que pode levar a perpetuação da lesão cerebral.

1.3.4. Mecanismos de Proteção Cerebral contra a Toxicidade derivada da Hemoglobina

A haptoglobina (Hp) e a hemopexina (Hx) são proteínas plasmáticas sintetizadas pelo fígado, e suas funções são a de ligar a hemoglobina e heme livres, respectivamente, que foram liberadas na hemólise intravascular e removê-las de circulação. Os complexos haptoglobina-hemoglobina são endocitados por macrófagos/microglia através do receptor CD163. Evidência recente sugere que Hp e Hx pode ter papéis na captação da hemoglobina e do heme no SNC após o AVEh. Zhao et cols mostraram que a expressão de Hp está aumentada na área peri-hematoma após o AVEh (Zhao et al., 2009). Além do transporte da Hp para o parênquima cerebral como resultado da disjunção da BHE, Hp pode ser sintetizada por oligodendrócitos, como demonstrado em experimentos de co-cultura neurônio-glia (Zhao et al., 2009). Além disso, os oligodendrócitos protegem os neurônios da toxicidade da Hb através da liberação de Hp, e camundongos hipohaptoglobinêmicos apresentam lesão cerebral mais grave e maior perda neuronal e lesão de substância branca quando comparados aos controles. Todos esses dados em conjunto sugerem que a Hp pode ser um componente importante da proteção do SNC através da captura da Hb. Entretanto, Galea et cols descreveram que a maior parte da Hb não está ligada à Hp, o que sugere que o sistema CD163-Hb-Hp está saturado e que a rota principal para o *clearance* de Hb do SNC é através da passagem livre de Hb através da BHE a favor de um gradiente de concentração (Galea et al., 2012). Além disso, pacientes com hipohaptoglobinorraquia apresentam uma incidência reduzida de infarto cerebral tardio (Galea et al., 2012). Esta evidência sugere que, embora a

secreção de Hp seja um mecanismo protetor contra a hemoglobina livre, sua magnitude e importância no contexto do AVEh ainda não está claramente estabelecida.

Hx é uma glicoproteína plasmática que é sintetizada por hepatócitos e também tem um papel no *clearance* do heme livre. A síntese de Hx no cérebro humano ainda é duvidosa, mas parece ser primariamente produzida por neurônios e induzida por ferro e heme (He et al., 2010). Entretanto, em um estudo post-mortem, a Hx não foi encontrada no interior dos neurônios, mas dos oligodendrócitos (Morris et al., 1993). A Hx se liga ao heme e forma um complexo heme-Hx, que é captada por macrófagos CD91 (Hvidberg et al., 2005). A formação dos complexos heme-Hx pode facilitar a remoção do heme pela microglia/macrófagos após o AVEh. Dados que suportem essa hipótese, entretanto, estão faltando. Em camundongos *knockout* para hemopexina, a viabilidade das células do *striatum* e a atividade locomotora três dias após a lesão era significativamente reduzida quando comparado ao grupo controle. Além disso, o conteúdo de heme tecidual havia aumentado 2,7x (Chen et al., 2011). A Hx também parece ser sintetizada pelos neurônios, e sua deleção resultou em aumento do volume do infarto e dos déficits neurológicos (Wang et al, 2008). Além disso, os complexos heme-Hx protegeram os neurônios da morte celular associada ao estresse oxidativo e induziram a expressão de heme-oxigenase 1 (HO-1). Hemopexina também reduziu a degradação e o acúmulo intra-neuronal de heme e aumentou a exportação de heme em 4x (Wang et al, 2006).

Embora exista um grande corpo de evidência implicando o ferro e o heme livres na fisiopatologia da lesão secundária à hemorragia intracraniana, este conhecimento não é facilmente traduzível para os trabalhos em humanos, principalmente para os ensaios clínicos. Da mesma forma, o papel dos mecanismos de proteção contra a hemoglobina e o heme ainda não está totalmente esclarecido. Considerando que os ensaios clínicos que tentaram limitar a lesão primária não foram bem sucedidos até o momento, a compreensão dos mecanismos de

lesão secundária tornou-se fundamental para estabelecer as bases que norteiem o desenvolvimento de pesquisas clínicas.

2. OBJETIVOS GERAIS E ESPECÍFICOS

2.1 – OBJETIVOS GERAIS

- Avaliar o papel dos produtos da degradação da hemoglobina e dos mecanismos de proteção contra a hemoglobina e heme na fisiopatologia do dano cerebral após a hemorragia cerebral

2.2 – OBJETIVOS ESPECÍFICOS

- Avaliar a cinética dos produtos de degradação da hemoglobina, hemopexina, haptoglobina e resposta inflamatória nos primeiros três dias após a hemorragia intracraniana;

- Analisar a relação entre a concentração de ferro, heme, hemopexina, haptoglobin e resposta inflamatória e a mortalidade precoce (7 dias);

- Correlacionar a concentração dos produtos de degradação da hemoglobina com a resposta inflamatória após o insulto hemorrágico.

3. METODOLOGIA

3.1 – DESENHO DO ESTUDO

Trata-se de uma coorte prospectiva de pacientes internados com hemorragia intracraniana com hemoventrículo (AVEh ou HSA), comprovada por tomografia computadorizada (TC) de crânio, e com necessidade de instalação de derivação ventricular externa (DVE) dentro de até 24 horas após o início dos sintomas. Foram incluídos pacientes internados nas unidades neurointensivas dos hospitais Copa D'Or (8 leitos), Quinta D'Or (10 leitos) e Hospital de Clínicas de Niterói (10 leitos). Esta coorte transcorreu de janeiro de 2008 até março de 2012. Os critérios de exclusão foram gravidez, idade < 18 anos e pacientes considerados com sobrevida inferior à 24 horas na admissão hospitalar. O estudo foi aprovado pelo Comitê de Ética em Pesquisa do Hospital Copa D'Or e do Instituto de Pesquisa Clínica Evandro Chagas sob os números 101/07 e 0057.0.009.009-11. O termo de consentimento informado foi obtido de todos os pacientes ou seus representantes legais (Anexo 1).

Após a inclusão no estudo, 10 mL de sangue eram coletados nos três primeiros dias de internação no CTI para análise de citocinas, produtos do metabolismo da hemoglobina, S100β e enolase, hemopexina e haptoglobina, e 2 mL de líquor através do sistema de DVE nos mesmos dias. Nessas datas também eram realizadas, pela rotina hospitalar, a dosagem de citometria geral e específica, glicose e proteína no líquor, além do cálculo do índice citométrico. O índice citométrico pode ser definido como: [(concentração de leucócitos no líquor/concentração de hemácias no líquor)/(concentração de leucócitos no sangue/concentração de hemácias no sangue)]. As amostras de líquor eram coletadas pela equipe de enfermagem das unidades nas quais os pacientes se encontravam internados nos dias já programados previamente pelo serviço para coleta de líquor de rotina para análise microbiológica.

As amostras de sangue e líquor eram processadas inicialmente nos hospitais. Tais amostras eram imediatamente centrifugadas, separadas em alíquotas e armazenadas a -70°C até o momento da análise, as quais foram realizadas nos seguintes laboratórios:

1) Laboratório de Imunofarmacologia – Departamento de Fisiologia e Farmacodinâmica da Fiocruz - análise de citocinas, hemopexina, haptoglobina e marcadores de lesão neuronal;

2) Laboratório de Pesquisa em Estresse Oxidativo – Instituto de Bioquímica Médica - UFRJ – análise dos produtos da degradação da hemoglobina

Os pacientes foram acompanhados até a alta hospitalar ou óbito. Analisamos a letalidade precoce (até 7 dias após o evento hemorrágico).

3.2 – AVALIAÇÃO CLÍNICA E ESCORES PROGNÓSTICOS

Os pacientes eram acompanhados clinica e laboratorialmente através de coleta dos seguintes dados (pior valor dentro do mesmo dia): pressão arterial, pressão intracraniana, pressão de perfusão cerebral, uso e dose de aminas vasopressoras, frequência cardíaca, frequência respiratória, uso de ventilação mecânica, temperatura axilar ou central, escala de coma de Glasgow e gases arteriais. Estes dados eram analisados também através de escores prognósticos: *Acute Physiology and Chronic Health Evaluation* (APACHE) II e *Simplified Acute Physiology Score* (SAPS) II no 1º dia de entrada no estudo. Para a estratificação da gravidade neurológica do evento inicial, utilizamos a escala de coma de Glasgow e as escalas de Fisher e Hunt-Hess para os pacientes com HSA. A evolução neurológica dos pacientes foi feita clinicamente através da escala de coma de Glasgow.

3.3 – AVALIAÇÃO POR IMAGEM

Todos os pacientes eram submetidos a tomografia computadorizada de crânio (TCC) com análise volumétrica do hematoma na admissão na emergência, 24 horas após e mais uma vez entre 10 a 14 dias de evolução. Esses procedimentos de imagem faziam parte da rotina do serviço.

3.4 – ANÁLISE DE CITOCINAS, PRODUTOS DA DEGRADAÇÃO DA HEMOGLOBINA E MECANISMOS DE PROTEÇÃO CEREBRAL

3.4.1 Ensaio de Citocinas Multiplex (Luminex®)

Os níveis de citocinas foram dosados no plasma e no líquor utilizando-se pares de anticorpos. O ensaio é realizado através a coleta de sangue, com separação de plasma por centrifugação (2000 rpm, por 15 minutos). Com o *kit* de citocinas (interleucina [IL] 1 beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, interferon [IFN] gama, *granulocyte colony-stimulating factor* [G-CSF], *granulocyte-macrophage colony-stimulating factor* [GM-CSF], *monocyte chemoattractant protein* [MCP] 1, *macrophage inflammatory protein-1* [MIP] beta, *tumour necrosis factor* [TNF] alfa e RANTES) foi realizado o ensaio de acordo com as instruções do fabricante (Bio-Rad, Hercules, CA, USA). De modo breve, amostras (50μL) diluídos em tampão para plasma foram colocadas em placas de 96 poços com filtro. As amostras foram incubadas com microesferas ligadas a anticorpo (50 μL em 2.000 esferas/poço) em temperatura ambiente por 30 minutos, em agitador de placa (300 rpm), em ambiente escuro; seguido de 3 lavagens com solução contendo anticorpos secundários (25μL/poço) adicionados aos poços e depois incubados em temperatura ambiente em agitador de placas, por mais 30 minutos. Estreptavidina-PE (16 μg/ml em tampão do ensaio) foi adicionada aos poços (50 μL) e incubada em temperatura ambiente por mais 10 minutos. Proteínas não associadas às microesferas foram filtradas pelos poços, usando vácuo

e nova lavagem com tampão foi realizada. Após a última lavagem, 125 µL de tampão foi adicionado em cada poço e nova incubação foi feita com agitador de placa por 1 minuto a 500 rpm e depois por 3 minutos a 300 rpm. A análise dos dados foi realizada de acordo com o software Bio-Plex Manager (Bio-Rad).

3.4.2- Produtos da Degradação da Hemoglobina

A determinação de ferro foi realizada através de um ensaio colorimétrico usando a ferrozina como descrito na literatura (Carter, 1976). Em resumo, em meio ácido, o ferro ligado à transferrina se dissocia em íon férrico que é convertido em íon ferroso através da ação do ácido tioglicólico. Com a adição do cromógeno ferrozine®, [ácido 3-(2-piridil)-5,6 bis (4-fenilsulfônico)-1,2,4-triazina], forma-se um complexo de cor rosa, cuja absorbância medida em 560nm é proporcional ao conteúdo de ferro da amostra.

Para a determinação dos níveis de heme, foi utilizado um ensaio colorimétrico (GenWay Biotech, San Diego, CA) que utiliza a atividade da peroxidase na presença do heme para realizar a conversão de uma sonda incolor em um componente colorido capaz de ser quantificado na leitura a 570nm. Pode-se detectar heme na faixa de 5-160pg (10-250 fmol).

3.4.3 - Mecanismos de Proteção Cerebral e Marcadores de Lesão Neuronal

As concentrações de hemopexina (Hx), haptoglobina (Hp), enolase and S-100β foram mensuradas utilizando-se kits comerciais de ELISA de acordo com as instruções do fabricante (LifeSciences, Newberg, OR). Neste ensaio, a Hx, Hp, enolase e S-100β presentes nas amostras reagiram com seus respectivos anticorpos, os quais foram adsorvidos a superfície de poços de polistireno. Então, esses anticorpos foram adicionados conjugados à peroxidase (HRP). A enzima ligada ao imunosorbente foi, então, mensurada em 450nm através da adição do substrato cromogênico, 3,3',5,5'-tetrametilbenzidina (TMB).

3.5 – ANÁLISE ESTATÍSTICA

Todas as variáveis numéricas foram expressas como mediana e intervalo interquartil (IQ 25-75%). Todas as variáveis numéricas foram testadas para verificar a distribuição dos valores (teste de normalidade). Nenhuma variável apresentou distribuição normal. Foram aplicados os testes de Mann-Whitney e Kruskal-Wallis para comparação entre 2 grupos e 3 ou mais grupos, respectivamente. Variáveis categóricas foram analisadas com o teste qui-quadrado ou teste de Fisher. Teste de Spearman foi utilizado para detectar correlações entre variáveis contínuas. Os dados foram colocados em planilha eletrônica (Microsoft Excel), para posterior análise estatística com o pacote estatístico SPSS 17.0 (SPSS Inc.) ou *GraphPad Prism* para Mac, versão 6.0 (GraphPad Software, San Diego, CA, USA).

Testamos as diferenças das concentrações de cada citocina, ferro, heme, hemopexina, haptoglobina, enolase e s-100β entre os dias 1, 2 e 3 após o evento hemorrágico (teste Kruskall-Wallis). Testamos também as diferenças entre as concentrações nos compartimentos liquórico e plasmático de cada citocina, ferro, heme, hemopexina, haptoglobina, enolase e S-100β (teste de Mann-Whitney). Posteriormente comparamos as concentrações entre sobreviventes e não-sobreviventes (desfecho em 7 dias). Correlacionamos as concentrações de ferro e heme nos três primeiros dias após a hemorragia intracraniana e das citocinas analisadas (teste de Spearman).

4. RESULTADOS

4.1. CARACTERÍSTICAS DOS PACIENTES

Quinze pacientes foram incluídos; a mortalidade em 7 dias foi de 40% (6 pacientes) e a mortalidade hospitalar foi de 73,3% (11 pacientes). Seis pacientes (40%) eram do gênero masculino e a mediana de idade foi de 59 anos (IQ 55-65). Dez pacientes (66,6%) foram admitidas por HSA e cinco, por AVEh (33,3%). As características clínicas e demográficas estão expressas na tabela 1.

Tabela 1 – Características demográficas e clínicas dos pacientes de acordo com o desfecho em 7 dias. Valores são expressos como mediana e intervalo interquartil.

Característica	**Todos os pacientes (n=15)**	**Sobreviventes (n=9)**	**Não-Sobreviventes (n=6)**
Gênero Masculino (%)	6 (40%)	6 (66,6%)	3 (50%)
Idade (anos)	59 (55-65)	62 (56,5- 65)	49 (38-72)
Escala de Coma de Glasgow	7 (6-9)	7 (4-14)	7 (5,75-10)
Escala de Hunt-Hess	4 (2,25-4)	4 (2-4,25)	3,5 (2,25-4)
SAPS II	43 (32-53)	37,5 (24,5-61)	44 (33-56,5)
Diagnóstico	AVC hemorrágico – 5 (33,3%) HSA – 10 (66,6%)	AVC hemorrágico – 3 (33,3%) HSA – 6 (66,6%)	AVC hemorrágico – 2 (33,3%) HSA – 4 (66,6%)
Ventilação Mecânica na Admissão no CTI	15 (100%)	9 (100%)	6 (100%)
Choque na Admissão no CTI	11 (73,3%)	6 (66,6%)	5 (83,3%)
Mortalidade em 7 dias	6 (40%)		
Mortalidade Hospitalar	11 (73,3%)		

SAPS II – *Simplified Acute Physiology Score* II

4.2. CINÉTICA DOS PRODUTOS DE DEGRADAÇÃO DA HEMOGLOBINA, DA HEMOPEXINA E HAPTOGLOBINA

Ao analisarmos a cinética dos produtos do metabolismo da hemoglobina nos compartimentos plasmático e liquórico durante os primeiros três dias após o evento hemorrágico, percebemos que há uma redução significativa da concentração de ferro no plasma 48h após a admissão, mantendo-se a concentração estável nas 72h após o evento (243,4 x 74,85 x 94,4 mg/dl; $p<0,05$). As concentrações de ferro no líquor e de heme tanto no plasma quanto no líquor mantém-se estáveis durante todo o período estudado.

Com relação à cinética da hemopexina e haptoglobina, podemos observar que suas concentrações mantém-se inalteradas durante os três primeiros dias após a hemorragia intracraniana, tanto no plasma quanto no líquor. Entretanto, ao compararmos os níveis liquóricos e plasmáticos de hemopexina e haptoglobina, notamos que a concentração de Hx e Hp no líquor é muito menor que no plasma e que ela não varia durante o curso do AVE. Esses dados sugerem que esses mecanismos de proteção contra a hemoglobina e o heme são insuficientes no SNC para exercer seu papel protetor. Os resultados da cinética dos produtos da degradação da hemoglobina, hemopexina e haptoglobina nos compartimentos plasmático e liquórico podem ser vistos na tabela 2.

Tabela 2 – Cinética do ferro, heme, hemopexina e haptoglobina plasmáticos e liquóricos nos três primeiros dias após o AVC hemorrágico. Valores estão expressos como mediana e intervalo interquartil. *p < 0,05

	24h	48h	72h
Plasma			
Ferro (mg/dl)	243,4 (137,8-459,1)	74,85 (53,04-244,9)*	94,4 (3,67- 167,3)
Heme (nM)	628 (587-1125)	604,7(583,4- 633,2)	630,7 (594,8- 658,8)
Hemopexina (mg/dl)	46,11 (25,02-78,47)	50,45 (17,16-113,8)	30,26 (15,46- 65,37)
Haptoglobina (mg/dl)	72,4 (42,9- 156,3)	109,3 (43,52- 245,5)	97,32 (59,18-205,5)
Líquor			
Ferro (mg/dl)	50,93 (34,01 – 73,62)	37,76 (32,92- 170,2)	54,99 (43,57-72,26)
Heme (nM)	599,9 (591,9-643,8)	613,5 (591,7-745,9)	682,4 (639,6-1093)
Hemopexina (mg/dl)	0,95 (0-8,0)	0 (0-2,82)	0,07 (0- 4,74)
Haptoglobina (mg/dl)	0,59 (0- 4,89)	0,86 (0-5,85)	1,27 (0- 6,17)

4.3. CINÉTICA DOS MARCADORES DE LESÃO CEREBRAL

A análise da cinética dos marcadores de lesão cerebral mostra um pico da concentração de enolase no líquor nas primeiras 24 horas após o AVE com redução gradativa subsequente de seus níveis nas 48 e 72 horas subsequentes. Por outro lado, a enolase plasmática sofre um aumento progressivo de sua concentração durante o período do estudo, atingindo o seu ápice 72 horas após o evento.

Surpreendentemente, não houve alterações na cinética de S-100β durante o período do estudo. Esses resultados podem ser vistos na tabela 3.

Tabela 3 – Cinética da enolase e S-100β plasmáticas e liquóricas nos três primeiros dias após AVC hemorrágico; unidade: mg/dl. Os valores estão expressos em mediana e intervalo interquartil. *p < 0,05

	24h	48h	72h
Plasma			
Enolase	2,65 (1,91- 8,17)	4,85 (3,31- 136,4)	38,06 (6,29- 99,56)*
S-100β	3,25 (2,28- 4,24)	3,4 (1,56- 6,15)	2,21 (1,62- 4,87)
Líquor			
Enolase	16,42 (3,68- 57,64)	4,24 (2,48- 11,91)	2,84 (1,6- 32,23)*
S-100β	3,63 (2,2- 6,48)	3,04 (2,36- 5,04)	3,24 (1,86- 4,46)

4.4. CINÉTICA DAS CITOCINAS

Apenas as citocinas com mais de 70% de recuperação foram avaliadas. Desta forma, analisamos as concentrações de IL-1b, IL-2, IL-6, IL-8, GM-CSF, IP-10, MCP-1, MIP-1a, MIP-1b e RANTES no plasma dos pacientes. Além disso, analisamos as concentrações de IL-1b, IL-2, IL-4, IL-6, IL-8, GM-CSF, IP-10, MCP-1, MIP-1a, MIP-1b, FGF e RANTES no líquor. Não houve diferença estatisticamente significativa tanto nas concentrações plasmáticas quanto liquóricas das citocinas durante os três dias de avaliação. Apenas a concentração liquórica de IL-8 apresentou um aumento em 48h quando comparado aos valores basais, retornando aos níveis iniciais 72 horas após o evento (23,7 x 246,8 x 37,65 pg/ml; $p< 0,05$). Esses resultados podem ser vistos nas tabelas 4 e 5.

Tabela 4 – Cinética das citocinas plasmáticas nos primeiros três dias após AVE hemorrágico; unidade: pg/ml. Os valores estão expressos em mediana e intervalo interquartil.* $p < 0,05$

	24h	48h	72h
IL-1b	4,03 (0,0527-7,115)	0,07 (0,01-6,04)	2,02 (0,07-6,04)
IL-2	17,67 (5-36,47)	17,67 (5,345-22,37)	17,67 (7,76-36,47)
IL-6	97,6 (0,001-287,2)	26,15 (0,001-342)	68 (19,61- 1098)
IL-8	32,08 (2,38-71,95)	12,25 (3,14- 31,23)	14,95 (3,34-94,32)
GM-CSF	43,87 (0,001- 423,1)	16,79 (5,45-146,4)	146,4 (43,87-694,6)
IP-10	553,6 (373,6-1395)	456,1 (144,4-875,9)	506,2 (226,7-3342)
MCP-1	36,48 (2,57-361,3)	62,18 (11,86-596,1)	41,51 (12,25-1087)
MIP-1A	16,98 (11,57- 28,09)	16,98 (9,93- 24,45)	20,11 (12,12- 27,38)
MIP-1B	35,77 (22,14-54,73)	20,03 (7,26-54,73)	35,77 (20,03-65,67)
RANTES	0,001 (0,001- 14,52)	0,001 (0,001- 195,8)	0,001 (0,001- 92,5)

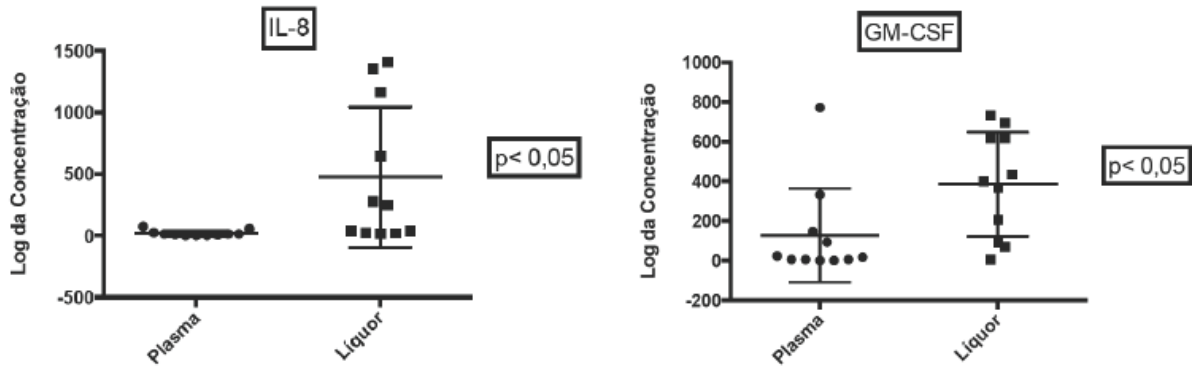
Tabela 5 – Cinética das citocinas no líquor nos três primeiros dias após AVC hemorrágico. Os valores estão expressos em mediana e intervalo interquartil; unidade: pg/ml. * p< 0,05

	24h	48h	72h
IL-1b	6,04 (0,001-15,3)	0,07(0,001-6,04)	2,5 (0,035-31,12)
IL-2	17,67 (5-47,98)	5 (1,33-26,35)	26,76 (17,67-63,42)
IL-4	0,001 (0,001- 34,98)	0,001 (0,001- 39,61)	0,001 (0,001- 34,98)
IL-6	157,4 (26,15-794,2)	109,84 (26,15-207,5)	109,84 (13,08-791,8)
IL-8	23,7 (3,83-91,93)	246,8 (23,7-1165)*	37,65 (17,11-860,9)
FGF	257,3 (96,09-445,7)	405,5 (96,09-877,5)	257,3 (129,8-1138)
GM-CSF	205,1 (48,82-639,9)	400,7 (91,58-618,21)	537,4 (43,84-964,4)
IP-10	1157 (538,7-3117)	1127 (337,3-1711)	1305 (419,3-1702)
MCP-1	352,2 (31,77-5326)	244,4 (31,77-653,7)	61,89 (16,98-598,6)
MIP-1a	14,14 (7,25- 23,52)	11,57 (7,25- 23,52)	23,52 (15,56- 51,28)
MIP-1b	27,47 (20,03-54,73)	35,77 (20,03-128,3)	22,14 (20,03-194,2)
RANTES	85,75 (10,81-1979)	129,6 (0,001-3036)	460,5 (9,49-3352)

4.5. COMPARAÇÃO ENTRE AS CONCENTRAÇÕES PLASMÁTICAS E LIQUÓRICAS DE CITOCINAS

Ao compararmos as concentrações de citocinas no plasma e no líquor, percebemos que, 48 horas após o evento hemorrágico, os níveis de IL-8 e GM-CSF são bastante superiores no líquor que no plasma (246,8 x 12,25 pg/ml e 400,7 x 16,79 pg/ml, respectivamente; $p< 0,05$), como pode ser visto na figura 2. Não há diferença estatisticamente significativa entre os compartimentos plasmático e liquórico das demais citocinas durante o período estudo. Esse resultado sugere haver uma compartimentalização da resposta inflamatória no SNC.

Figura 1 – Comparação entre concentrações plasmáticas e liquóricas de GM-CSF e IL-8. Os valores estão expressos em log das concentrações. Unidade: pg/ml.



4.6. DETERMINANTES DE MORTALIDADE PRECOCE (7 DIAS)

Ao compararmos a citometria global e diferencial e o índice citométrico, bem como as concentrações de glicose e proteína do líquor de sobreviventes ao dos não-sobreviventes 7 dias após o evento hemorrágico, podemos perceber que o líquor dos não-sobreviventes apresenta um perfil claramente mais inflamatório 72h após a hemorragia intracraniana que o dos sobreviventes. Esses dados podem ser vistos na tabela 6.

As concentrações de ferro e heme no plasma 48h após o evento são estatisticamente maiores nos não-sobreviventes que nos sobreviventes (496,04 x 58,5 mg/dl e 624,3 x 584,7 nM, respectivamente; $p< 0,05$). Esses dados sugerem que a quantidade total de ferro sistêmico está relacionada ao pior prognóstico em pacientes com hemorragia intracraniana. Os resultados podem ser vistos nas tabelas 7 e 8.

Não há diferença estatisticamente significativa em relação aos níveis de hemopexina e haptoglobina tanto no plasma quanto no líquor quanto observamos a mortalidade em 7 dias.

Tabela 6 - Citometria e bioquímica do líquor 72h após AVE hemorrágico de acordo com o desfecho em 7 dias. Valores são expressos como mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
Hemácias (contagem/mm^3)	13250 (3815-18406,25)	17685 (9619,5-34652,5)
Citometria (contagem/mm^3)	6 (5-9,25)	237 (140-1078)*
Polimorfonucleares (contagem/mm^3)	0 (0-0)	58 (18,75-680,25)*
Linfócitos (contagem/mm^3)	5 (3,5-6,5)	179 (121,25-397,75)*
Glicose (mg/dl)	69 (57,75-77,25)	100 (36,5-106)
Proteína (mg/dl)	58,55 (44,47-92,2)	54(38,75-64,75)
Índice Citométrico	0.0009 (0.0003- 0.005)	0.03 (0.01- 0.05)*

Tabela 7 - Concentrações de ferro e heme plasmáticos após o AVC hemorrágico de acordo com o desfecho. Valores estão expressos como mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
24h		
Ferro (mg/dl)	424,08 (137,52-607,44)	273,44 (148,22-360,94)
Heme (nM)	617,9 (583,6- 1125)	669,9 (592,7- 1562)
48h		
Ferro (mg/dl)	58,5 (53,04-74,85)	496,04 (208,18-905)*
Heme (nM)	584,7 (578,4- 621,7)	624,3 (604,7- 7445)*
72h		
Ferro (mg/dl)	102,79 (64,81-192,87)	256,54 (207,66-756,35)
Heme (nM)	610 (591,7- 999,9)	615,6 (588,7- 654,1)

Tabela 8 - Concentrações de ferro e heme liquóricos após o AVC hemorrágico de acordo com o desfecho. Valores estão expressos como mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-sobreviventes (n=6)
24h		
Ferro (mg/dl)	41,9 (10,1-50,93)	71,31 (39,43-84,86)
Heme (nM)	613,9 (596,8-643,8)	592,3 (582,7-793)
48h		
Ferro (mg/dl)	37,76 (34,9-59,13)	35,04 (32,99-57,44)
Heme (nM)	610 (591,7- 999,9)	615,6 (588,7- 654,1)

Em relação a concentração de citocinas, podemos perceber que, nas primeiras 24h após o evento, os níveis plasmáticos de IL-2 e RANTES foram significativamente maiores nos não-sobreviventes que nos sobreviventes (36,47 x 5,0 pg/ml e 1718 x 4,64 pg/ml, respectivamente; $p< 0,05$). Já 72h após a hemorragia intracraniana, as concentrações plasmáticas de IL_6 e IL-8 são marcadores de mortalidade precoce (26,15 x 1271 pg/ml; $p< 0,05$ e 134,8 x 3,83 pg/ml, $p< 0,05$; respectivamente. Não há diferença estatisticamente significativa entre sobreviventes e não-sobreviventes com relação as demais citocinas plasmáticas. Esses dados sugerem que a resposta inflamatória sistêmica parece ser um fator determinante para a sobrevida dos pacientes após o evento hemorrágico cerebral.

No líquor, os níveis de IL-4 nas primeiras 24 horas após a hemorragia intracraniana são maiores nos sobreviventes que nos não-sobreviventes (34,98 x 0,001 pg/ml; $p<0,05$). Não houve diferença significativa entre sobreviventes e não-sobreviventes em relação as demais citocinas no líquor. Os resultados podem ser vistos nas tabelas 9 a 13.

Em relação aos marcadores de morte neuronal e astrocitária, não houve diferença em relação aos níveis tanto plasmáticos quanto liquóricos de enolase e S100-β entre os sobreviventes e não-sobreviventes durante o período estudado.

Tabela 9 - Concentrações das citocinas plasmáticas nas primeiras 24 horas após AVE hemorrágico de acordo com o desfecho; unidade: pg/ml. Os valores estão expressos em mediana e intervalo interquartil.* $p < 0,05$

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
IL-1b	0,07 (0,001-8,19)	6,04 (3,05-40,06)
IL-2	5,0 (0,001-22,22)	36,47 (11,34-90,37)*
IL-6	0,001 (0,001-376,2)	109,8 (13,08 - 211,8)
IL-8	1,9 (0,001-56,2)	53,81 (13,77-71,95)
GM-CSF	43,81 (0,001-694,6)	92,18 (24,66-300)
IP-10	373,6 (0,001-682,5)	792,1 (407-2503)
MCP-1	3,77 (0,001-47,04)	150,8 (11,52-2765)
MIP-1A	9,41 (0,42-21,89)	20,11 (15,56-42,6)
MIP-1b	31,62 (5,53-35,77)	54,73 (20,03-106,3)
RANTES	4,64 (0,001 - 73,81)	1718 (230,6-6433)*

Tabela 10 - Concentrações das citocinas plasmáticas nas primeiras 48 horas após AVE hemorrágico de acordo com o desfecho; unidade: pg/ml. Os valores estão expressos em mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
IL-1b	0,001 (0,001-6,04)	6,04 (1,56-12,99)
IL-2	5,46 (0,001-17,67)	17,67 (17,67-31,77)
IL-6	2,34 (0,001-207,5)	226,4 (6,53-3739)
IL-8	12,25 (0,001-12,25)	13,77 (1,78-46,28)
GM-CSF	16,79 (0,001-146,4)	48,82 (5,45-602,6)
IP-10	373,6 (11,68-792,1)	456,1 (159-21754)
MCP-1	51,25 (2,01-150,8)	358,6 (5,40-1464)
MIP-1A	11,57 (1,71-16,98)	23,52 (18,62- 26,3)
MIP-1b	20,03 (5,01-54,73)	14,02 (2,00- 31,84)
RANTES	66,61 (0,67- 1198)	977,7 (0,001- 2252)

Tabela 11 - Concentrações das citocinas plasmáticas nas primeiras 72 horas após AVE hemorrágico de acordo com o desfecho; unidade: pg/ml. Os valores estão expressos em mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
IL-1b	0,07 (0,07- 6,04)	6,04 (1,51- 4815)
IL-2	14,1 (3,75 - 22,37)	27,07 (5,41- 2552)
IL-6	26,15 (0,001 – 109,8)	1271 (250,7 - 4180)*
IL-8	3,83 (0,001 - 17,64)	134,8 (19,16-4062)*
GM-CSF	92,18 (0,001- 267,3)	582,8 (186,9- 2283)
IP-10	305,5 (0,001 - 506,2)	5145 (561,3- 9860)
MCP-1	15,08 (3,77- 333,3)	955,9 (14,45- 3510)
MIP-1A	16,98 (5,43- 22,89)	23,52 (5,88- 2468)
MIP-1b	27,9 (6,0- 54,73)	48,32 (20,03- 2284)
RANTES	1266 (3,58- 1861)	1283 (81,04- 3668)

Tabela 12 - Concentrações das citocinas liquóricas nas primeiras 24 horas após AVE hemorrágico de acordo com o desfecho; unidade: pg/ml. Os valores estão expressos em mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
IL-1b	6,04 (1,51- 34,4)	0,001 (0,001- 6,04)
IL-2	17,67 (1,25- 40,40)	5,0 (0,001- 32,83)
IL-4	34,98 (0,001- 89,36)	0,001 (0,001- 0,001)*
IL-6	133,6 (26,15- 762,5)	26,15 (0,001- 610,4)
IL-8	8,04 (0,001- 154,5)	23,7 (6,12- 72,87)
GM-CSF	300 (205,1- 1083)	92,18 (2,72- 356,1)
IP-10	1687 (414,9- 5700)	698,3 (152,7- 2062)
MCP-1	192 (5,4- 3221)	278,8 (105,8- 13263)
MIP-1A	15,56 (7,25- 52,17)	7,25 (2,74- 17,55)
MIP-1b	20,03 (20,03- 33,7)	27,47 (10,02- 114,1)
RANTES	85,75 (43,25-2519)	85,75 (0,001- 1884)
FGF	257,3 (24,02- 857,3)	96,09 (0,001- 257,3)

Tabela 13 - Concentrações das citocinas liquóricas nas primeiras 48 horas após AVE hemorrágico de acordo com o desfecho; unidade: pg/ml. Os valores estão expressos em mediana e intervalo interquartil. *p < 0,05

	Sobreviventes (n=9)	Não-Sobreviventes (n=6)
IL-1b	0,07 (0,001- 6,04)	4,27 (0,62- 212,4)
IL-2	1,33 (1,33- 17,67)	22.01 (8,16- 109,4)
IL-4	0,001 (0,001- 0,001)	19,81 (0,001- 103,4)
IL-6	109,8 (0,001- 794,2)	68,0 (26,15- 152,6)
IL-8	275,3 (17,64- 1165)	142,2 (27,19- 1076)
GM-CSF	435,6 (205,1- 694,6)	229 (26,98- 555,2)
IP-10	1451 (946- 3010)	641,6 (84,32- 1347)
MCP-1	244,4 (31,77- 653,7)	331,1 (38,1- 959)
MIP-1A	11,57 (7,25- 23,52)	15,39 (4,79- 100,1)
MIP-1b	35,77 (20,03- 128,3)	50,55 (35,77- 113,9)
RANTES	3,58 (0,001- 4186)	147,1 (64,85- 2234)
FGF	445,7 (96,09- 877,5)	250,8 (24,02- 1318)

4.7. CORRELAÇÃO ENTRE FERRO E HEME E CITOCINAS PLASMÁTICAS E LIQUÓRICAS

Ao analisarmos a correlação entre ferro e heme e concentração das citocinas, percebemos que há uma correlação negativa entre os níveis plasmáticos de ferro 24h após o evento e a concentração plasmática de IP-10 72h após a hemorragia intracraniana (r= -0,67; p= 0,025), Curiosamente, também há uma correlação forte positiva entre os níveis liquóricos de ferro 48h após o ictus e os níveis de IP-10 no líquor 72h após o evento (r=0,97; p=0,03).

Com relação ao heme, observou-se uma correlação forte entre os níveis de heme no SNC nas 24h após a hemorragia intracraniana e a concentração liquórica de MIP-1b 48 h após o ictus (r=0,76; p=0,01). Ainda no SNC, a concentração de heme nas primeiras 48 após o evento está correlacionada negativamente com os níveis de MCP-1 72h após o ictus (r=-0,82; p=0,03). Esses dados sugerem que o ferro e o heme podem ter um papel em iniciar a resposta inflamatória no SNC e no compartimento sistêmico após o evento hemorrágico cerebral. Não houve correlação estatisticamente significativa entre o ferro e o heme e as demais citocinas no período estudado.

5. DISCUSSÃO

No nosso trabalho, pudemos observar que: 1) Ferro e heme estão correlacionados ao desencadeamento da resposta inflamatória e a maior concentração de ferro sistêmico está associada a pior prognóstico em pacientes com hemorragia intracraniana; 2) A defesa contra a hemoglobina e o heme no SNC é insuficiente, e não varia durante os três primeiros dias do curso da doença; 3) Perfil inflamatório tanto no plasma quanto no líquor no terceiro dia após o ictus está associado a maior mortalidade, enquanto um perfil anti-inflamatório no primeiro dia após o evento parece ser protetor; 4) A cinética dos biomarcadores de lesão cerebral sugere que existe uma morte neuronal preferencial logo após o ictus com subsequente extravasamento de antígenos do compartimento liquórico para o sistêmico; 5) Após a hemorragia intracraniana, há o desencadeamento de resposta inflamatória cerebral com aumento dos níveis liquóricos de IL-8 e concentrações de IL-8 e GM-CSF no líquor cerca de 20x superiores as do plasma.

Nós observamos que o ferro e heme séricos estão associados a maior mortalidade 7 dias após o evento hemorrágico e que existe correlação entre as concentrações de ferro e heme no líquor e a resposta inflamatória cerebral, através da liberação de IP-10 e MIP-1b. Um dos mecanismos pelo qual o ferro pode levar ao dano cerebral é através da amplificação da resposta inflamatória. Tem sido demonstrado que células da microglia, quando repletas de ferro, apresentam maior liberação de MMP-9, TNF- α e IL-1β quando expostas ao LPS comparadas as que estavam depletadas de ferro (Zhang et al., 2006). Além disso, aumento das concentrações de ferro em cultura de oligodendrócitos levou à ativação de NF-κβ (Zhang et al., 2006).

O heme, por sua vez, também parece contribuir com a resposta inflamatória após o evento hemorrágico cerebral. Heme estimula diretamente TLR-4 através da via de sinalização do MyD88/TRIF, levando a produção de IL-6, TNF- α e IL-1β . Também já foi

descrito que o heme estimula a migração e ativação de leucócitos, principalmente neutrófilos, através da expressão de moléculas de adesão nas células endoteliais (ICAM-1, VCAM-1 e E-selectina), aumento da permeabilidade vascular e aumento da expressão e secreção de quimiocinas (Graça-Souza et al., 2002). Heme também estimula a secreção de IL-8 no cérebro (Lou et al, 2009). Esses dados sugerem que tanto o ferro quanto o heme podem atuar como fatores indutores de inflamação no SNC após a hemorragia intracraniana.

No nosso estudo, a concentração de ferro no líquor estava fortemente relacionada à concentração de IP-10 também no líquor. Já os níveis de heme estavam relacionados ao de MIP-1b no SNC. O IP-10, um membro da família das quimiocinas, é quimioatrativa para monócitos e linfócitos T humanos e promove a adesão de linfócitos T às células endoteliais. Já o MIP-1b também é uma quimiocina produzida por macrófagos (Sherry et al., 1988). O MIP-1b ativa granulócitos (neutrófilos, eosinófilos e basófilos) e induz a síntese e liberação de citocinas pró-inflamatórias tais como IL-1, IL-6 e TNF- α pelos macrófagos. A elevação dos níveis de IP-10 já foram descritos no líquor nas meningites virais, pacientes com HIV (Yuan et al., 2013) e esclerose múltipla (Sørensen et al., 2002). IP-10 também já foi relacionado a fisiopatologia da hemorragia intracraniana. Em um estudo em cultura de glia humana, IFN induziu a liberação de IP-10 (Smith et al., 2013). Trombina também estimular a liberação de IP-10 (Simmons et al., 2013).

A relação temporal entre as concentrações de ferro e heme e as de IP-10 e MIP-1b parecem sugerir uma relação causal. Entretanto, outros fatores confundidores podem estar relacionados à liberação de IP-10 e MIP-1b, como a própria trombina, enquanto as concentrações de ferro e heme seriam apenas marcadores da extensão da hemorragia.

De forma interessante, o heme apresentou uma correlação negativa com os níveis de MCP-1 no líquor. A proteína quimiotática para monócitos-1 (MCP-1/CCL2) é uma CC quimiocina, com atividade sobre monócitos, células T, células NK, basófilos e mastócitos. Foi

originalmente identificada como um mediador do recrutamento e ativação monocitária (Yoshimura et al., 1989) Em adição à suas propriedades quimiotáticas, relatos recentes sugeriram que esta quimiocina ativa células da linhagem monocítica, aumentando a expressão da molécula de adesão CD11b/CD18. Níveis séricos elevados de MCP-1 já foram relacionados ao pior prognóstico em crianças com encefalopatia hipóxico-isquêmica (Jenkins et al., 2012) e aumento de MCP-1 no tecido cerebral está relacionado a apoptose neuronal em um modelo de TCE (Liu et al., 2013). Trombina, ferro e heme já foram caracterizados como indutores da secreção de MCP-1, o que , de certa forma, contradiz os resultados do nosso trabalho (Wenzel et al., 1995), (Kanakiriya et al., 2003), (Johnson et al, 2010). Entretanto, todos esses estudos foram realizados em relação a secreção de MCP-1 no plasma, o que sugere que a relação entre ferro e heme e MCP-1 no SNC pode ser diferente do compartimento sistêmico.

Outro aspecto interessante é a correlação entre ferro e heme plasmáticos e a mortalidade precoce neste grupo de pacientes. A sobrecarga sistêmica de ferro já foi descrita como um fator de pior prognósticos em pacientes com AVE. Ossa et cols mostraram, em uma coorte de pacientes que AVE hemorrágico, que a ferritina sérica na admissão hospitalar era um fator independente de mau prognóstico em pacientes com hemorragia intracraniana (Ossa et al., 2010). Nesse estudo também foi observado que não havia qualquer correlação entre os níveis de ferritina na admissão e os de outros marcadores de fase aguda (como leucocitose, glicose sérica e fibrinogênio), mostrando que a ferritina, neste caso, era um marcador dos estoques sistêmicos de ferro, e não apenas relacionada a própria doença aguda. Outros trabalhos também mostraram que a ferritina sérica está associada tanto à progressão do AVE em pacientes com AVE isquêmico (AVEi) (Dávalos et al., 2000) quanto ao pior prognóstico e transformação hemorrágica em pacientes com AVEi tratados com Rt-PA (Millan et al., 2007).

Com relação aos possíveis mecanismos de defesa do SNC contra os produtos do metabolismo da hemoglobina, nosso trabalho mostrou que a concentração de hemopexina e haptoglobina no líquor após o evento hemorrágico é bastante reduzida, e seus níveis não variam ao longo dos três primeiros dias após o ictus. Nossos resultados contradizem os estudos experimentais que mostram um papel proeminente da Hx e da Hp na proteção cerebral contra os efeitos deletérios dos produtos da degradação da hemoglobina. Zhao et cols mostraram que a expressão de Hp encontra-se aumentada na região peri-hematoma após AVCh (Zhao et al., 2009). Além disso, um dos mecanismos de proteção neuronal exercida pelos oligodendrócitos contra a toxicidade da hemoglobina se dá através da liberação de Hp e camundongos hipohaptoglobinêmicos apresentam lesão cerebral mais extensa, perdas neuronais e de substância branca maiores e piora do déficit neurológico quando comparado a camundongos controle (Zhao et al., 2009). Por outro lado, camundongos que tem expressão aumentada de Hp são menos vulneráveis à lesão cerebral após o AVEh (Zhao et al., 2009). O sulforano, que é um agente ativador de Nrf2, já se mostrou capaz de aumentar as concentrações plasmáticas e cerebrais de Hp e, com isso, reduzir a lesão cerebral após o AVEh em modelos experimentais (Zhao et al., 2007).

Em relação ao papel da hemopexina, já foi descrito que, em camundongos *knockout* para Hx, três dias após o evento hemorrágico a viabilidade das células do *striatum* é significativamente menor, o conteúdo de heme tecidual é 2,7x maior e a atividade motora é bastante reduzida comparada aos camundongos *wild-type* (Chen et al., 2011). A inibição da síntese de hemopexina pelos neurônios resulta em aumento do volume do infarto e dos déficits neurológicos, e a formação de complexos heme-Hx foi um fator protetor de morte celular por estresse oxidativo, além de induzir a liberação de HO-1(Li et al., 2009).

Entretanto, a tradução destes achados experimentais para humanos não é tão bem definida. Em pacientes sépticos, níveis séricos aumentados de haptoglobina se mostraram

protetores (Janz et al., 2013). A despeito de estudos em humanos que mostraram que a hipohaptoglobinorraquia e a presença do genótipo Hp 2-2 (com menor capacidade de ligação à hemoglobina) estão associadas a maiores taxas de déficit cerebral tardio (Galea et al., 2012) e vasoespasmo (Borsody et al., 2006), outros autores, como Galea et al, observaram que o sistema CD163-Hp-Hb encontra-se saturado em pacientes com HSA e que a principal rota de saída da Hb do SNC é através de difusão pela BHE a favor de um gradiente de concentração (Galea et al., 2012). Nossos achados corroboram o fato de que, em humanos, a capacidade de defesa cerebral contra a hemoglobina e o heme é bastante reduzida e, portanto, não parece exercer um papel fundamental na prevenção da mortalidade precoce.

Outro achado interessante foi que o perfil pró-inflamatório, tanto do ponto de vista sistêmico como no líquor, estava associado a mortalidade precoce, enquanto um perfil anti-inflamatório no líquor era protetor. Sabe-se que a resposta inflamatória, tanto no SNC, quanto sistêmica, está relacionada a mortalidade precoce. A ativação da resposta imunológica sistêmica após a HSA e o AVEh está amplamente documentada e frequentemente se manifesta por níveis elevados de citocinas, as quais são as principais efetoras da inflamação sistêmica (Gruber et al., 2000).

A síndrome da resposta inflamatória sistêmica (SRIS), originalmente descrita em associação à sepse (Bone et al., 2009), pode ser vista em associação a um grande número de insultos não-infecciosos, como trauma e cirurgia . A SRIS é um processo sistêmico associado a ativação e disfunção endotelial (Aird et al, 2003) que altera a perfusão tecidual, promove a disfunção orgânica e piora o prognóstico. A SRIS é vista em até 60% dos pacientes com HSA e é associada a disfunção orgânica extra-cerebral e pior prognóstico (Gruber et al., 1999)(Tam et al., 2010). Seus componentes, como febre e leucocitose, estão relacionados a eventos adversos após a HSA (Oliveira-Filho et al., 2001)(McGirt et al., 2003) e após o AVEh (Agnihotri et al., 2011). Tanto níveis elevados de pressão intracraniana (Graetz et al., 2010)

como a ativação simpática (Gao et al., 2009) parecem atuar como gatilhos para a liberação sistêmica de IL-6 após a HSA.

A IL-6 é bom preditor de desenvolvimento de SDOM e de mortalidade hospitalar em pacientes com trauma grave e sepse (Pinsky et al., 1993) (Frink et al., 2009). A IL-6 apresenta cinética de aumento até 2-3 dias após o início de quadro de SIRS/sepse (Oda et al., 2005). Pacientes não-sobreviventes permanecem com níveis maiores de IL-6 do que os sobreviventes, e ainda existe correlação desta citocina com o escore SOFA. Em pacientes com HSA, os níveis plasmáticos de IL-6 parecem refletir, não apenas a gravidade da lesão cerebral inicial, como também o curso e prognóstico da doença (Muroi et al., 2013). O nosso trabalho corrobora os achados da importância da IL-6 nos pacientes com hemorragia intracraniana, uma vez que as concentrações de IL-6 no plasma 72 horas após o evento hemorrágico em pacientes não-sobreviventes era significativamente maior que nos sobreviventes (1271 x 26,15 pg/ml).

Estudos sugerem que, após o AVEh, vários genes pró-inflamatórios estão estimulados, incluindo fatores de transcrição, citocinas, quimiocinas, proteases extracelulares e moléculas de adesão (Lu et al., 2006). Em pacientes com AVEh, níveis séricos de IL-6 aumentam no primeiro dia após o evento e persistem elevados até o sétimo dia (Kim et al., 1996). Os níveis plasmáticos de IL-6 nas primeiras 24h também estão correlacionados a magnitude do edema cerebral subsequente (Castillo et al., 2002) e à expansão do hematoma (Silva et al., 2005).

A IL-8 também é um importante mediador da resposta inflamatória sistêmica. Estudos clínicos demonstraram um aumento nos níveis da IL-8 no soro de pacientes sépticos (Oberholzer et al., 2005) (Bozza et al., 2007) e nos pulmões de pacientes com a Síndrome da Angústia Respiratória Aguda (SARA) (Wiedermann et al., 2004). Em pacientes portadores de TCE, níveis séricos elevados de IL-8 tem sem mostrado repetidamente associados ao pior

prognóstico (Gopcevic et al., 2007), (Mussack et al., 2002), (Kushi et al., 2003), (Lo et al, 2010).

Após a hemorragia intracraniana, inicia-se uma pronunciada resposta inflamatória local, com recrutamento de neutrófilos periféricos, ativação de astrócitos e microglia e liberação de mediadores inflamatórios (Wang et al, 2007). Em nosso trabalho, observamos que existe um aumento pronunciado de IL-8 no líquor após o evento hemorrágico e que um padrão de resposta inflamatória no SNC, representado pelo aumento da citometria global e específica 72h após a hemorragia intracraniana, também está associado ao pior prognóstico. Em humanos, IL-8 é detectada em níveis bastante baixos no líquor em condições normais. Em pacientes com TCE, a IL-8 parece alcançar seu ápice precocemente, chegando a níveis tão altos como 29000 pg/ml (Kushi et al., 2003), e níveis aumentados de IL-8 estão associados a maior mortalidade. Em crianças, o aumento da concentração de IL-8 no líquor é da mesma magnitude do aumento em crianças com meningite bacteriana (Whalen et al., 2000). Os níveis de IL-8 tanto em adultos quanto em crianças estão relacionados a gravidade da disfunção da BHE (Kossmann et al., 1997) e ao aumento da mortalidade (Whalen et al., 2000). Nestes estudos, a concentração de IL-8 é significativamente maior no líquor que no plasma, sugerindo que a origem desta quimiocina é a produção intratecal de IL-8, o que está de acordo com nossos resultados. A IL-8 também é quimiotática para neutrófilos e parece regular a pleocitose liquórica em modelos de meningite pneumocócica (Ostergaard et al., 2000).

O GM-CSF também foi encontrado em concentrações cerca de 20x superiores no líquor que no plasma nos nossos pacientes. GM-CSF é uma citocina pró-inflamatória que é expressada no SNC por neurônios, astrócitos e microglia (Franzen et al, 2004). GM-CSF também é secretada pelo endotélio vascular, cruza a BHE e pode ser detectada no SNC (Coxon et al, 1999). Em estudos realizados no tecido cerebral de pacientes que morreram vítimas de TCE grave, esta citocina encontrava-se estimulada de 6-122h após a lesão (Frugier

et al., 2010), e ela é encontrada em concentrações maiores no líquor de pacientes com sofreram TCE associado a hipóxia secundária (Yan et al., 2014). De forma interessante, além de suas ações pró-inflamatórias (como estímulo a quimiotaxia e fagocitose de neutrófilos), o GM-CSF parece ter efeito neuroprotetor em um modelo de TCE (Shultz et al., 2014).

A resposta inflamatória local exacerbada está associada a maior mortalidade precoce dos pacientes em nossa coorte. Os níveis de leucócitos, polimorfonucleares e linfócitos são significativamente maiores nos não-sobreviventes que nos sobreviventes. Pleocitose liquórica já foi descrita em pacientes com hemorragia intraventricular, tendo seu pico sido observado no sétimo dia após o evento (Hallevi et al., 2012). Em outro trabalho, o líquor de pacientes com HSA se mostra predominantemente pró-inflamatório no segundo dia após o sangramento, levando ao aumento do número de leucócitos recrutados e ao aumento da permeabilidade vascular a partir do sexto dia, com quebra da BHE (Schneider et al., 2012). Nosso trabalho corrobora os achados de aumento importante da pleocitose já no terceiro dia após a hemorragia intracraniana, o que pode indicar precocemente os pacientes que irão evoluir de forma desfavorável.

Da mesma forma, o perfil anti-inflamatório predominante, caracterizado pelos maiores níveis de IL-4, está associado à sobrevida em 7 dias. IL-4 é uma citocina que induz a diferenciação de linfócitos T *helper naive* (Th0) em células Th2. A presença de IL-4 em tecidos extravasculares promove a ativação de macrófagos em células M2 e inibe a ativação clássica de macrófagos em células M1. O aumento de macrófagos M2 está associada a secreção de IL-10 e TGF-β, resultando na diminuição da inflamação patológica. Em um modelo experimental, camundongos *knockout* para IL-4 apresentaram volumes de área infartada maiores, desfecho neurológico pior e atividade espontânea reduzida quando comparados aos camundongos controle (Xiong et al., 2011). IL-4 também se mostrou eficaz em aumentar os efeitos neuroprotetores da secreção de tiol e lactato e em inibir a liberação de

citocinas Th1 (Garg et al, 2009). Nosso estudo encontra-se em concordância com os trabalhos que mostraram uma associação entre um perfil anti-inflamatório no líquor e melhor prognóstico após a lesão cerebral aguda (Bell et al., 1997).

Entretanto, não existe consenso sobre o papel das citocinas predominantemente anti-inflamatórias e o desfecho dos pacientes com lesão neurológicas agudas. Outros estudos realizados em pacientes com TCE mostraram relação entre os níveis liquóricos de IL-10 e maior mortalidade (Kirchhoff et al., 2008) e pior prognóstico funcional (Shiozaki et al., 2005). Vale lembrar que o meio anti-inflamatório (IL-4, IL-10 e TGF-β) está relacionado à diferenciação da microglia para um fenótipo apresentador de antígenos (ativação adaptativa) em detrimento de um fenótipo fagocítico (ativação inata), o que pode perpetuar a lesão cerebral a longo prazo e contribuir para o pior desfecho (Town et al, 2005).

Biomarcadores de lesão cerebral, tais como a enolase específica do neurônio (NSE) e S100-β, originárias dos neurônios e dos astrócitos, respectivamente, tem o potencial de auxiliar na detecção precoce e na quantificação da gravidade da lesão cerebral aguda bem como no prognóstico. Em nosso trabalho, a concentração de enolase no líquor atingiu um pico cerca de 24h após o evento e diminuiu nos dias subsequentes, enquanto a concentração plasmática de enolase aumentou continuamente nas primeiras 72 horas após o evento. Não houve nenhuma alteração estatisticamente significativa tanto dos níveis séricos quanto liquóricos de S100-β.

A enolase e a S100-β tem sido tradicionalmente descritos como marcadores importantes de lesão do SNC após a hemorragia intracraniana. Em pacientes portadores de HSA, já foi descrita a detecção de enolase e S100-β no líquor e no plasma nos três primeiros dias após o ictus (Kacira et al., 2007). Em pacientes com AVEh, Brea et al analisaram sequencialmente os níveis séricos de enolase e S100-β e valores de pico de ambos os biomarcadores foram encontrados 24h após o evento (Brea et al., 2009). A cinética

encontrada dos marcadores de lesão cerebral pode ser representativa de morte neuronal preferencial e liberação dos antígenos neuronais no sangue. Em um modelo canino de HSA, os neurônios compreendiam cerca de 80% das células parenquimatosas cerebrais que morreram, embora tenha sido reportada tanto perda neuronal quanto de astrócitos (Sabri et al., 2008). Esse achado foi confirmado em outro estudo no qual morte celular por apoptose foi ativada dentro de 10 minutos após a HSA, e a maioria das células apoptóticas era de origem ou neuronal ou vascular (Friedrich et al, 2012). A hipótese da perda neuronal preferencial é corroborada por diversos estudos que mostram que o neurônio é mais vulnerável aos efeitos tóxicos da hemoglobina e do heme (Lara et al., 2009), do ferro (Kress et al, 2002), ao estresse oxidativo gerado pela bilirrubina não-conjugada (Brito et al., 2008) e a privação de glicose (Muneer et al., 2011) quando comparado aos astrócitos. Outra explicação para o pico de enolase no líquor poderia ser a inserção da DVE (Brandner et al., 2013); entretanto, ela não explica a liberação preferencial de enolase em detrimento da de S100-β.

A quebra da BHE já foi descrita em várias condições clínicas, tais como TCE (Korn et al., 2005), AVCi (Strbian et al., 2008), epilepsia (Tomkins et al., 2008) e sepse (Bozza et al., 2010). Consequências da quebra da BHE são a formação de edema (Bozza et al., 2010) e liberação de antígenos do parênquima cerebral para o sangue (Yan et al., 2012). A liberação de antígenos para a periferia pode explicar o aumento progressivo dos níveis séricos de enolase, espelhando o decréscimos dos níveis liquóricos.

Nosso estudo tem algumas limitações, tais como o pequeno número de pacientes estudados e a heterogeneidade dos mesmos. Entretanto, os pacientes estudados foram submetidos a análise sequencial do padrão de resposta inflamatória e do comportamento dos produtos de degradação da hemoglobina e dos mecanismos de proteção cerebral contra a hemoglobina e o heme tanto no compartimento sistêmico quanto no SNC. Essa análise sequencial tem sido pouco estudada na literatura. Além disso, nosso trabalho fornece

evidência preliminar do papel do ferro e do heme no estímulo à resposta inflamatória no SNC e da insuficiência de mecanismos de proteção contra os produtos da degradação da hemoglobina do cérebro humano. Nosso trabalho também reforça a noção que a SIRS é um fator importante no desfecho dos pacientes com hemorragia intracraniana. Estudos clínicos mais amplos são necessários para definir o papel desses biomarcadores no AVEh e na HSA.

6. CONCLUSÕES

1) Ferro e heme estão correlacionados à maior resposta inflamatória e a maior concentração de ferro sistêmico está associada a pior prognóstico em pacientes com hemorragia intracraniana;

2) Estes mecanismos de proteção cerebral contra a hemoglobina e o heme (como hemopexina e haptoglobina) no SNC são bastante reduzidos, e não variam durante os três primeiros dias do curso da doença;

3) Perfil inflamatório tanto no plasma quanto no líquor no terceiro dia após o ictus está associado a maior mortalidade, enquanto um perfil anti-inflamatório no primeiro dia após o evento parece ser protetor;

4) Após a hemorragia intracraniana, há o desencadeamento de resposta inflamatória cerebral com aumento dos níveis liquóricos de IL-8 e concentrações aumentadas de IL-8 e GM-CSF no líquor em relação as do plasma;

5) A cinética da enolase sugere que existe uma morte neuronal preferencial logo após o ictus com subsequente extravasamento de antígenos do compartimento liquórico para o sistêmico.

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ANEXO 1

Termo de Consentimento

Título

Resposta Inflamatória Secundária ao Acidente Vascular Encefálico Hemorrágico – Análise de Citocinas, Produtos do Metabolismo do Heme e Marcadores de Estresse Oxidativo

Justificativa e objetivos

O derrame cerebral acontece quando uma veia do cérebro se rompe e o sangue passa de dentro da veia para o cérebro. Quando o paciente apresenta um derrame cerebral, o organismo produz diversas substâncias para ajudar a eliminar o sangue de dentro do cérebro. Essas substâncias, entretanto, também podem prejudicar a recuperação cerebral. Nosso objetivo nesse estudo é o de estudar melhor essas substâncias e, com isso, entender como se faz a eliminação de sangue do cérebro e o processo de destruição cerebral provocado pelo sangue.

Proposta do Estudo

O Sr(a) _____ está sendo convidado a participar deste estudo, para estudar a presença de substâncias produzidas pelo nosso organismo em resposta ao derrame cerebral.

Explicação dos Procedimentos

Será realizada a coleta de uma amostra de sangue de 10 (dez) mL, através de uma punção de veia, utilizando-se material estéril e descartável. Este procedimento é semelhante a coleta de sangue para exames laboratoriais de rotina. Será coletado nos dias 1, 3, 5, 7, uma vez entre os dias 10-14 e na alta após início do estudo e na alta do CTI. Eventualmente, em pacientes com derrame, ocorre a formação de coágulos que impedem a circulação do líquido (chamado líquor) entre as metades do cérebro. O líquor se acumula e aumenta a pressão

dentro do cérebro, o que leva a necessidade de instalação de um cateter para a drenagem do líquor. Nos pacientes que necessitem da colocação desse cateter (chamado derivação ventricular externa – DVE), rotineiramente no CTI coletamos 2mL de líquor em dias alternados para a monitorização da presença de possíveis infecções relacionadas a presença do cateter de DVE. Nestes dias da coleta rotineira do líquor, coletaremos mais 2mL para os procedimentos do estudo.

Benefícios

No seu caso não há benefícios diretos, mas este estudo poderá ajudar a entender melhor os mecanismos ligados ao derrame cerebral.

Desconfortos e Riscos

Os desconfortos que podem ocorrer são aqueles relacionados a uma retirada normal de sangue para exame, como dor no local da punção venosa e formação de um hematoma local. Como já mencionado anteriormente, a coleta de líquor pelo cateter de DVE já é realizada de rotina para fins de assistência, independente da participação no estudo, não implicando em qualquer risco adicional. Este estudo não implica em riscos, nem em qualquer modificação do tratamento empregado ou administração de medicamentos experimentais.

PARTICIPAÇÃO VOLUNTÁRIA NO ESTUDO

A participação neste estudo é voluntária. Você pode se recusar a participar, bem como cancelar sua participação a qualquer momento do estudo. Esta decisão não afetará de nenhuma maneira os cuidados médicos que lhe serão oferecidos.

CONFIDENCIALIDADE

O seu nome não será mencionado em publicações ou relatórios produzidos para este estudo. Entretanto seu prontuário médico poderá ser consultado pelos profissionais envolvidos no estudo.

SE VOCÊ TEM DÚVIDAS

Se você tiver qualquer dúvida sobre o estudo, por favor telefone para a Dra. Cássia Righy Shinotsuka no telefone 2545-3412 ou Dr. Fernando Bozza no telefone 2598-4492

(ramal 244).

CONSENTIMENTO PARA A PARTICIPAÇÃO NO ESTUDO

A sua assinatura significa que você leu este formulário ou que ele foi lido para você, que lhe foram dadas todas as explicações sobre o estudo, que você recebeu respostas para as suas dúvidas, está satisfeito com as informações que lhe foram dadas e concordou com a participação no estudo.

_____ _____

Assinatura (Paciente) Data

Se o paciente não é capaz de consentir:

A sua assinatura, como representante legal do paciente, significa que você leu este formulário ou que ele foi lido para você, que lhe foram dadas todas as explicações sobre o estudo, que você recebeu respostas para as suas dúvidas, está satisfeito com as informações que lhe foram dadas e concordou com a participação do paciente no estudo.

_____ não é capaz de dar o seu consentimento.

Nome do Paciente

(em letra de forma)

_____ _____

Nome do Representante Legal Grau de parentesco com o paciente

(em letra de forma)

_____ _____

Assinatura (Representante legal) Data

ANEXO 2

Artigo de revisão: "Molecular, cellular and clinical aspects of intracerebral hemorrhage: are the enemies within?" a ser submetido.

Molecular, cellular and clinical aspects of intracerebral hemorrhage: are the enemies within?

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Keywords: iron, heme, hemorrhagic stroke, subarachnoid hemorrhage, intracranial bleeding, inflammatory response, reactive oxygen species

Abstract

Hemorrhagic stroke is a disease with high incidence and mortality rates. In addition to the mass lesions that result from hemorrhagic stroke, substances such as iron and heme can induce a potent inflammatory response and exert a direct toxic effect on neurons, astrocytes, and microglia. In the present review, we discuss the mechanisms of brain injury secondary to hemorrhagic stroke, focusing on the involvement of the hemoglobin derived products (HDP) heme and iron as major players of cellular redox imbalance, inflammation and glutamate excitotoxicity. Potential natural mechanisms of protection against free hemoglobin and heme such as haptoglobin and hemopexin, respectively are highlighted. We finally discuss the experimental and clinical trials targeting free iron and heme scavenging as well as inflammation, as potential new therapies to minimize the devastating effects of hemorrhagic stroke on brain structure and function.

Introduction

Each year, 795,000 people have strokes in the United States, or 1 person every 40 seconds, on average. These incidents account for approximately 103,000 cases of hemorrhagic stroke each year in the United States and 2 million cases worldwide (1) . Intracerebral hemorrhage (ICH) is a deadly disease, with an estimated mortality rate of approximately 40% within 1 month following an event, and only 12-39% of the survivors are able to perform activities of daily living at the time of hospital discharge (1). Ten years following the first stroke, only approximately 24% of patients will still be alive (2). In the coming years, ICH cases are expected to grow, due to the aging of the population and the increasing use of anticoagulants and thrombolytics, which currently account for 20% of brain hemorrhage cases in the United States (3). Thus, understanding the pathophysiology of brain injury after ICH is of pivotal importance for developing new therapeutic approaches that can reduce these high morbidity and mortality rates.

After the massive release of blood within the brain parenchyma, red blood cell (RBC) lysis begins almost immediately, releasing free heme and iron into the CNS. Free heme and decreased hemopexin (Hx) levels have already been associated to increased mortality in severe sepsis (4), as well as to the severe systemic manifestations of malaria (5). Iron is also being increasing studied as a major component in neurodegenerative diseases, such as Alzheimer and Parkinson's (6). In brain hemorrhage, many experimental studies have clarified the toxic effects of heme and iron upon brain tissue and some pre-clinical trials have evaluated iron chelation, stimulation of antioxidant capacity, and anti-inflammatory therapies as potential therapies after hemorrhagic stroke. There is some evidence in humans that corroborates experimental studies;

clinical trials testing iron chelation therapy and anti-inflammatory drugs are on the way. In the present manuscript, we review the mechanisms of ICH brain injury secondary to the release of hemoglobin-derived products (HDPs) within brain parenchyma, the protective systems, as well as the experimental studies and clinical trials aiming reduction of HDP-related toxicity to the CNS.

Mechanisms of Brain Injury

The hemoglobin derived products (HDP)

A number of studies have implicated hemoglobin (Hb), heme and iron as key mediators of brain injury. Erythrocyte lysis occurs within minutes and continues for several days following hematoma formation, releasing hemoglobin and other toxic substances (iron and heme) into the brain parenchyma (7), (8), (9). Once in the extracellular milieu, hemoglobin is eventually digested and converted into heme and then to biliverdin, carbon monoxide and iron by heme oxygenases (HO). Hemoglobin, heme and iron are potent cytotoxic molecules that, through a wide array of mechanisms, potentiate the inflammatory response (10) and are also potent pro-oxidant components that oxidize proteins, nucleic acids, carbohydrates and lipids, disrupting cell signaling with multiple celullar consequences summarized on Figure 1 (11), (12) .

Several lines of evidence converge to the fact that blood and HDP play a central role on the pathogenesis of ICH-associated injury. For example, infusion of packed erythrocytes induced edema and caused neurological deficits several days following injury, which suggests a role for erythrocyte lysis in delayed brain damage (13). However, infusion of lysed erythrocytes results in brain edema, blood brain barrier (BBB) disruption, and DNA injury within 24 hours, indicating that HDP exert toxic effects in the CNS (14), (15). Indeed, free Hb is not only a pro-oxidant molecule,

generating highly reactive hydroxyl radicals in a ascorbate-dependent process (16), but also cause CNS damage (17). Hb is also able to interact with nitric oxide (NO) (18), which cause hemoglobin iron oxidation (19) and NO decomposition into nitrate (NO_3^-) (20). NO consumption by free hemoglobin not only mediates vascular hypertension (21) but also seems to be directly involved on vasoconstriction in subarachnoid hemorrhage (22), (23). These evidence seem to explain the acute hypertensive response observed upon massive intravascular hemolysis (24), (25). Hb carbonylation can lead to heme release from hemoglobin. Free hemoglobin can be oxidized to methemoglobin, which is a key element in LDL oxidation. Heme also catalyzes LDL oxidation (26), promoting further tissue injury (27). Indeed, in vitro experiments demonstrated that neurons and astrocytes are sensitive to the toxic effects of free hemoglobin and heme, respectively, and, apparently, its nocive effects are also mediated by iron-independent mechanisms (28).

Iron is an essential element, and is utilized in a wide array of biochemical reactions in the CNS, such as neurotransmitter metabolism, myelin synthesis, and in cellular energy transduction reactions. Concentrations of brain iron are highest at birth, decrease during the first 2 weeks of life, and increase throughout life (29), (30), suggesting that the brain capacity of dealing with iron overload decreases with age. Iron toxicity to the CNS is mediated by various mechanisms, being the most important one through redox imbalance due to its capacity to generate hydroxyl radical by the classical Fenton reaction. Thus, the antioxidant mechanisms in the brain are consumed to ROS production and by decreasing the antioxidant defences. Sadrzadeh and colleagues have shown that iron and hemoglobin catalyzed hydroxyl radical production and lipid peroxidation (16), (17), (31). High levels of protein carbonyl were detected in the perihematomal white matter within minutes following autologous blood injection (32).

In fact, redox imbalance can persist for up to three days following injury, as demonstrated by the increased dihydroethidium staining (a marker for redox imbalance detected *in situ*) in the peri-ICH region (33). Reductions in superoxide dismutase (SOD) activity and increased DNA fragmentation following ICH were also described (15). In addition to their role in direct injury to cell membranes, ROS can activate the transcription factors NF-kβ (34) and activator protein-1 and can also induce BBB disruption, worsening the brain edema (35). ROS can also depress mitochondrial function (36) Iron can further propagate oxidative injury by inhibiting the enzymatic function of base excision repair pathway for DNA damage and by delaying the repair of DNA in cultured neurons (37).

To date, there has been only one clinical study that has examined ROS production as a mediator of brain injury following ICH. Mantle and colleagues found oxidized proteins in perihematomal brain tissue samples following hematomal drainage in 10 patients (38). However, evidence of ROS production was also found in the control samples (patients submitted to brain tumor resection or aneurysm clipping). It was hypothesized that the control patients were also subjected to higher levels of oxidative stress due to their underlying pathology (brain cancer and intracranial aneurysms). Despite the lack of clinical evidence, abundant experimental data show that ROS generation is a key component of brain injury following ICH.

Another mechanism of brain injury by iron is by amplifying inflammatory response. Lipopolysaccharide (LPS)- activated microglia loaded with iron had increased release of MMP-9 (39), TNF-α, and IL-1β than non-loaded microglia (40). Culture media from activated microglia was toxic for oligodendrocytes; this effect was reversed by iron chelation (40). Increases in iron levels also led to activation of NF-κβ (40).

Glutamate excitotoxicity seems to be an important mechanism in neuronal and oligodendrocyte death. Glutamate promote iron uptake in rat spinal cord explants (41) and, on the other hand, iron may mediate the toxic effects of glutamate and stimulate glutamate release by increasing aconitase activity (42), which is important for both glutamate synthesis, and energy metabolism. Glutamate can also increase BBB permeability and promote brain edema through NMDA receptors, which are stimulated by oxidative stress and inhibited by iron chelation (43), (44), (45). Furthermore, increasing evidence suggests that iron can induce neurodegeneration, promote neuronal autophagy (46), enhance the neurotoxicity of β-amyloid through transglutaminase expression (47), and cause neuronal atrophy and death (48). In humans, serum ferritin (49) and hematoma iron content (50), as measured by MRI, were associated to perihematomal edema development.

Hemoglobin and heme can also directly contribute to brain injury. Heme is a compound containing a Fe atom within its protoporphyrin ring. Ferrous (Fe^{+2}) is neutral however, ferric heme (Fe^{+3}) is positively charged and can bind anions (12) Free heme, which is heme not contained within hemoproteins, can act as a potent cytotoxic pro-oxidant compound and lead to oxidative stress (51). Due to its highly hydrophobic nature (12). heme can readily intercalate in cell membranes. Free heme can also oxidize LDL particles, which are cytotoxic to endothelial cells (26). This hypothesis is also supported by the fact that pharmacological antioxidants can confer cytoprotection against free heme (52).

In addition to direct stimulating oxidative stress, heme also participates in the inflammatory reaction by directly stimulating TLR4 (11), (53) or amplifying the inflammatory effects of microbial molecules (54) as can be seen in figure 2. Heme can also induce neutrophil migration, decomposition of organic radicals into highly reactive

alkoxyl and peroxyl radicals (55) and secretion of IL-8 (56) and TNF-α [52]. Furthermore, heme appears to induce the expression of pro-inflammatory adhesion molecules, both in vitro (57) and in vivo (58), and to induce increased vascular permeability (56), perpetuating brain edema. Besides promoting inflammatory reaction within the CNS, heme was also found to induce programmed cell necrosis in macrophages in vivo (38). Furthermore, neurons were found to be more sensitive to the toxic effects of heme (59) and hemoglobin (28) than astrocytes (figure 3), which could further perpetuate brain injury. Recent studies in mice demonstrated a critical role of TLR4 on the pathogenesis of hemolytic and hemorrhagic conditions (60).

Mechanisms of Brain Protection against HDP Toxicity

In the setting of severe hemolysis, several protective mechanisms are activated reducing the deleterious effects of free iron, heme, and hemoglobin. The main protective mechanisms consist on heme degradation by the heme-oxygenases into iron, carbon monoxide, and biliverdin, as well as hemoglobin and heme scavenging by haptoglobin (Hb) and hemopexin (Hx), respectively. While these mechanisms are well described in hemolytic diseases, such as malaria and other hemolytic anemias, their role in brain protection after hemorrhagic stroke is less clear. Compounds that upregulate the expression of antioxidants, like Nrf2 and PPAR-γ , also play a role in cerebral protection after intraparenchymatous bleeding.

Haptoglobin and Hemopexin

Haptoglobin (Hp) and hemopexin (Hx) are plasma proteins that are synthesized by the liver, and their functions are to bind free hemoglobin and heme, respectively, that have been released during intravascular hemolysis and to remove them from

circulation. Haptoglobin-hemoglobin complexes are uptaken by macrophages/microglia through the scavenger receptor CD163 . Recent evidence suggests that Hp and Hx may play roles in hemoglobin and heme scavenging in the CNS following ICH. In this sense, Zhao and colleagues have shown that Hp expression is increased in the perihematomal area following ICH (61). In addition to Hp transport to the brain parenchyma as a result of BBB disruption, Hp can be synthesized by oligodendrocytes, which was demonstrated in neuron-glial co-culture experiments (61). Furthermore, oligodendrocytes protect neurons from Hb toxicity through Hp release, and hypohaptoglobinemic mice experienced more extensive brain damage, neurological deficits, neuronal loss, and white matter injury following ICH compared to controls (61). These results suggest that Hp may be an important component of CNS protection by hemoglobin chelation. However, Galea and colleagues reported that most Hb was not bound to Hp, which suggests that the CD163-Hb-Hp system is saturated and that the primary route for Hb clearance from the CNS is freely crossing the BBB through a concentration gradient (62). Moreover, hypohaptoglobinorrhachia patients, which exhibit more effective clearance of Hb, have been associated with a reduced incidence of delayed cerebral infarct (DCI) (62). This evidence suggests that, although Hp secretion is a protective mechanism against free hemoglobin, its magnitude and importance after hemorrhagic stroke are not clearly established. The main components of brain protection against blood extravasation and drugs tested to enhance the mechanisms of protection are summarized in figure 4.

The Hp genotype may play a role in brain protection following ICH. There are three major Hp genotypes: Hp1-1, Hp1-2, and Hp2-2. Hp2-2 is the genotype with the least Hb-scavenging capacity (63). In a mouse model of SAH, the Hp 2-2 genotype was critical to the development of severe vasospasm (64). The stimulation of NO release

with diethylenetriamine resulted in significant increases in the basilar artery lumen patency of Hp 2-2 mice (65), as did the systemic administration of L-citrulline, which increases NO synthesis (66). In patients with SAH, Hp2-2 also appears to be associated with higher rates of vasospasm (67).

Hx is a plasmatic glycoprotein that is synthesized by hepatocytes playing a central role in heme scavenging. Hx binds to heme and forms a heme-Hx complex, which is cleared by CD91 macrophages (68). The synthesis of Hx in the human brain is unclear, but it appears to be primarily produced in neurons. In a neural culture, Hx synthesis was induced by heme (69). A post-mortem study found Hx immunostaining in neurons but not in oligodendrocytes (70). The formation of heme-Hx complexes may facilitate heme removal by microglia/macrophages following ICH. Data supporting this hypothesis, however, is scarce. In hemopexin knockout mice, the striatal cell viability three days following injury was significantly reduced, heme tissue content was 2.7-fold increased, and locomotor activity was reduced compared to wild-type mice (71). Deletion of Hx resulted in increased infarct volumes and neurological deficits (72). Moreover, heme-Hx complexes protected neurons from oxidative stress-associated cell death and induced the expression of heme-oxygenase 1 (HO-1) (73). Hx also decreased intra-neuronal heme accumulation and decreased heme breakdown (73). While Hx seems to have an important protective role against oxidative stress within the brain parenchyma, clearly, more studies are required to clarify the role of Hx in ICH.

Heme Oxygenase

Heme oxygenase (HO) is a rate-limiting enzyme of physiological heme degradation that catalyzes the conversion of heme into biliverdin, carbon monoxide, and iron. There are two isoforms of HO: HO-1 and HO-2. HO-2 is constitutively expressed and can be found in most cell types (including neurons), whereas HO-1 is induced

following ICH in microglia/macrophages (72), (74). HO-1 expression reaches its peak at 3 and 7 days following brain hemorrhage (75). In an autopsy study, HO-1 expression began approximately 2 h following ICH, peaked within 17-30 h and declined after 10 days (76).

The role of HO activity in ICH is controversial. The genetic deletion of HO-2 led to neurons that were more vulnerable to heme toxicity, a 30% increase in brain volume injury on day 1 and a 67% increase on day 3, and worsened neurological function (74). HO-2 knockout mice were more susceptible to brain damage following ICH, had increased neutrophil infiltration, microglial/macrophage and astrocyte activation, DNA damage, peroxynitrite production, and cytochrome c immunoreactivity. On the other hand, HO-1 null mice had reduced brain injury, neurological dysfunction, leukocyte infiltration and microglial activation, and a lower susceptibility to DNA damage. In animal models of Alzheimer and Parkinson's diseases, HO-1 expression promoted intracellular oxidative stress, the opening of the mitochondrial permeability transition pore, and the accumulation of non-transferrin iron in the mitochondrial compartment (77), (78). However, HO-1 knockout astrocytes demonstrated a 20-25% death rate and a fourfold increase in protein oxidation (79). Moreover, the increased sensitivity to heme toxicity observed in HO-2 knockout mice was reduced when HO-1 expression was stimulated with adenoviral gene transfer (80). The prevention of heme accumulation at intracellular toxic levels and the production of the antioxidants biliverdin/bilirubin may partially explain this protection (78), (81). HO-1 also stimulated the secretion of carbon monoxide, which appeared to play a protective role in a rat model of ICH (82).

One of the possible explanations for these competing results is that, whenever there is a transient rise of intracellular free heme, HO-1 synthesis is triggered. HO-1 removes free heme from the system in exchange for free reactive iron. Free iron, before

being sequestered from the intracellular medium by ferritin, can catalyze oxidative reactions. Moreover, in the long-term, depletion of free iron by the increase in ferritin pool may lead to a cellular desensitization to oxidant challenge (11).

Nrf2 and Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ)

Nrf2 is a transcriptional factor that stimulates the transcription of antioxidant genes, including quinine oxidoreductase 1, glutathione S transferase, glutamate-cysteine ligase, glutathione peroxidase, and HO-1 (83). Nrf2 is present in neurons, astrocytes, and microglia and is generally regarded as being neuroprotective. Shah et al demonstrated that Nrf2-/- mice were more prone to stroke damage than control mice following ischemia-reperfusion injury and that tert-butylhydroquinone, an Nrf2 inducer, attenuated neuronal death (84). In a collagenase model of ICH, Nrf2-deficient mice were more prone to severe neurological deficits, and a worsening of brain injury was associated with increases in leukocyte infiltration, ROS production, DNA damage, and cytochrome c release (85), (86). The depletion of Nrf2 increased the inflammatory reaction through the NF-κB pathway, stimulating the expression of TNF-α, IL-1β, IL-6, and MMP-9 (87).

However, Nrf2 expression is up-regulated in the endothelial and arterial smooth muscle cells (88), and the elevated expression of Nrf2 runs a parallel course with the development of vasospasm (89). Melatonin and erythropoietin appear to protect against early brain injury by stimulating the Nrf2-pathway (90), (91). These studies suggest that Nrf2 expression is neuroprotective against early inflammatory brain injury in hemorrhagic stroke models but may also play a role in vasospasm development. Further studies are required to clarify this issue.

PPAR-γ is another transcriptional factor that regulates the expression of two important antioxidant genes: catalase and superoxide dismutase. Zhao and colleagues have shown that the intrahemorrhage injection of 15D-PGJ_2 leads to the increased

expression of PPAR-γ and catalase in neurons and microglia (92), (93). PPAR-γ also reduced the expression of the pro-inflammatory genes TNF-α, IL-1B, MMP-9, and iNOS, the extracellular H_2O_2 levels, and prevented neuronal damage. These effects were parallel to stimulation of phagocytosis by microglia (92), (93). PPAR-γ has also been associated with reduced neurological dysfunction and hematoma resolution (92), (93). Supporting these evidence, rosiglitazone, an agonist of PPAR-γ, attenuated MPO activity and the expression of IL-1β and TNF-α (94). This compound also reduced oxyhemoglobin-induced TLR4 expression and TNF-α release in a culture of vascular smooth muscle cells (95), as well as reduced cerebral vasospasm following SAH by impairing TLR4 signaling (96). In summary, PPAR-γ seems to exert a protective role after hemorrhagic stroke by reducing inflammatory response and by increasing antioxidant protection. Thus, PPAR-γ agonists rise as potential drugs for clinical trials.

Experimental and Clinical Trials

Based upon evidence of iron and heme-induced brain injury and the protection mechanisms, many experimental and clinical trials have focused on three primary targets to reduce iron-mediated neuronal injury: iron scavenging, inhibition of inflammatory reaction induced by iron and heme, and the enhancement of natural protection pathways, like increasing expression of haptoglobin and hemopexin.

As a transition metal, iron is chemically defined by bearing incomplete electron orbitals, which is an essential feature of any free radical. This mean that as a free radical, iron present on the most abundant biological oxidation forms ($^{+2}$ and $^{+3}$) are naturally unstable species, which may react with nearest molecules to reach their chemical stability. Deferoxamine is an iron chelator that has been used in clinical practice for many years and it is able to bind iron in both free and complexed forms, with high

affinity, forming stable less reactive complexes. As a result, iron complexation by deferoxamine prevents cellular redox imbalance and neuronal death (97), (98). Deferoxamine also reduced hemoglobin-induced DNA damage, hippocampal neuronal death, brain atrophy and swelling (99). In addition to its role in iron chelation, deferoxamine has also been shown to reduce HO-1 expression in aged rats (100), attenuate the accumulation of 8-OHdG, a marker of nucleic acid oxidation, and enhance the secretion of APE/Ref-1, a DNA repair mechanism for oxidative damage (101). In a recent meta-analysis of pre-clinical trials, deferoxamine was found to improve neurobehavioral scores at the last point of assessment and to reduce brain edema (102). However, further studies were unable to find an association between deferoxamine administration and improved neurological outcomes in rat models (103), (104). Both studies that found negative results for deferoxamine were performed in collagenase-induced rat models of brain hemorrhage, raising questions regarding differences among the different models of ICH and their ability to accurately reproduce clinical practices.

Recently, the safety and tolerability of deferoxamine in ICH patients were investigated. Twenty patients were enrolled, and doses between 7 mg/Kg and 62 mg/Kg per day were tested. The primary side effect was mild hypotension, and the investigators concluded that deferoxamine was well tolerated and safe in the clinical setting (105). Further studies to establish the potential role of deferoxamine in preventing neurological dysfunction following ICH are eagerly awaited.

Minocycline is a tetracycline type of antibiotic, which has been shown to have neuroprotective properties in addition to its antibiotic, anti-inflammatory, anti-apoptotic, and antioxidant effects, which appear to play a beneficial role following the ICH (106), (107). Power et al reported that minocycline infusion inhibited IL-1α and MMP-12, diminished microglial activation and neutrophil infiltration in the brain,

reduced the appearance of apoptotic cells, and improved neurobehavioral outcomes in a mouse model (108). Another study showed that the systemic administration of minocycline reduced perihematomal brain edema, neurological deficits, and brain atrophy (106). However, other investigators have reported that, while minocycline had beneficial effects in reducing brain edema, microvessel loss, neutrophil infiltration, and TNF-α and MMP-12 secretion (107), it had no protective effects on striatal tissue and neuron loss. Xue and colleagues, in an autologous-blood mice model, suggested that a high dose of locally applied minocycline with intravenous supplementation might result in better neuronal protection following ICH (109). In a recent meta-analysis, minocycline was found to improve neurobehavioral outcomes only when infused for at least 24 hours following ICH (102). All this data together suggest that minocycline may have a beneficial effect after hemorrhagic stroke mainly by reducing inflammatory response. However, the use of minocycline to minimize the onset and progression of neurodegenerative diseases has been questioned. A phase III trial with 412 amyotrophic lateral sclerosis patients showed that minocycline was associated with non-significant trends of a faster decline in functional scores and higher mortality rates (110). In models of Parkinson's and Huntington's diseases, minocycline was also found to be associated with worst functional scores and increased neuronal loss (111). These trials cast a shadow of doubt on the effectiveness of minocycline in the treatment of neurological diseases, and further studies are required to clarify this issue.

Statins are inhibitors of hydroxymethylglutaryl-CoA reductase, and their primary effect is to reduce cholesterol biosynthesis and, therefore, to diminish blood cholesterol levels. However, in addition to their hypocholesterolemic effect, statins have pleiotropic properties (112), including improving endothelial function, attenuating vascular and myocardial remodeling, and inhibiting vascular inflammation and oxidation. These

pleiotropic effects are being studied in a wide variety of diseases, including ICH. In a rat model, atorvastatin administration 24 hours following ICH reduced cell loss in the striatum and improved neurobehavioral outcomes (113). These effects were attributed to increased synaptic plasticity, decreased expression of iNOS, and neutrophil/microglia recruitment, leading to a reduced inflammatory reaction (114). Statin was found to reduce brain water content, block neuron apoptosis, and reduce the plasmatic level of MMP-9 (115). Simvastatin reduced cognitive dysfunction (116) and diminished IL-1β secretion, while enhancing TGF-β release and TGF-β positive lymphocyte infiltration in the subarachnoid and perivascular spaces (117). Therefore, it was hypothesized that a statin-induced Th2 shift could provide neuroprotection. In a mouse model, simvastatin was also found to recouple eNOS and, therefore, prevent eNOS monomer formation, decrease superoxide radical production, and increase NO expression, leading to decreased vasospasm and neuronal injury (22).

Despite the beneficial effects observed in animal models, clinical studies have shown conflicting results regarding the association between prior statin use and mortality rates. Naval and colleagues demonstrated that prior statin use was associated with decreased perihematomal edema and mortality rates following ICH (118), (119). However, FitzMaurice et al found no association between statins and neurological outcomes or mortality rates (120). More recently, a meta-analysis with more than 2,000 patients with ICH demonstrated an association between prior statin use and good outcomes, as well as reduced mortality rates (121). However, an analysis of a Canadian registry with 2,466 patients found no association between preadmission statin use and outcomes in ICH (122), which makes the effects of prior statin use unclear. In face of the conflicting evidence provided by retrospective studies, clinical trials evaluating the effect of statin administration after hemorrhagic stroke were proposed. A pilot trial of

rosuvastatin in 75 ICH patients found better outcomes in the rosuvastatin group at discharge (123), and a simvastatin trial was closed due to poor enrolment (NCT00718328).

Sulphoraphane, a known Nrf2 activator, reduced oxidative damage and neurological deficits in animals (85). 15D-PGJ_2, a natural PPAR-γ agonist, increased the expression of catalase, primarily in neurons and microglia, following ICH (92), (93). Other PPAR-γ agonists that are already in clinical use include the thiazolidinediones, specifically pioglitazone and rosiglitazone, and a clinical trial of pioglitazone in ICH patients is current ongoing (124).

Conclusions

Brain injury following ICH is a complex phenomenon that involves systemic and local inflammatory reactions, direct toxicity of HDPs, free radical formation and, ultimately, cell death. A deeper insight into the mechanisms involved in ICH is required, as are clinical trials on new drugs that may help decrease the morbidity and mortality rates of this deadly disease.

Abbreviations:

15D-PGJ_2 - 15-Deoxy-Delta-12, 14-prostaglandin J2
BBB – blood brain barrier
CNS – central nervous system
DCI – delayed cerebral infarct
H_2O_2 – Hydrogen peroxide
Hb – Hemoglobin
HO – heme oxygenase
Hp - Haptoglobin
Hx - hemopexin
ICH – intracerebral hemorrhage
IL- interleukin
LPS – Lipopolysaccharide
MMP- Matrix metallopeptidase
MPO - myeloperoxidase
MRI – magnetic resonance imaging
NF-κβ - nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA- N-methyl-D-aspartate receptor

NO – nitric oxide
Nrf2 – Nuclear factor-like 2
PPAR- γ - Peroxisome proliferator-activated receptor gamma
RBC – red blood cells
ROS – reactive oxygen species
SAH – subarachnoid hemorrhage
TGF-β – transforming growth factor- β
TLR4 – toll-like receptor 4
TNF-α- tumor necrosis factor alpha

Competing Interests:

The authors state no competing interests.

Author's contributions:

CRS designed and drafted the manuscript; MTB and MFO helped draft the manuscript and revised it critically; FAB conceived the review, helped draft the manuscript, and gave final approval of the version to be published. All authors read and approved the final manuscript.

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ANEXO 3

Artigo original: "Hemoglobin metabolism byproducts trigger inflammatory response in patients with hemorrhagic stroke" a ser publicado

Title: Hemoglobin metabolism byproducts trigger inflammatory response in patients with hemorrhagic stroke

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Abstract: Introduction: In recent years, it has been increasingly recognized that iron and heme play key roles in the pathophysiology of brain injury after intracerebral and subarachnoid hemorrhage. However, these mechanisms have not been fully characterized in clinical studies.

Material and Methods: We conducted a prospective cohort of patients with intracerebral and subarachnoid hemorrhage. We assayed plasma and cerebrospinal fluid in the first three days after hemorrhagic stroke for iron, heme, hemopexine, haptoglobine, enolase, S-100β and cytokines. We analyzed the kinetics of these parameters and their relationship with early mortality.

Results: Hemopexine and haptoglobine concentrations are almost negligible in the brain after the event. Iron and heme levels are correlated with inflammatory response in the CNS and plasmatic and CSF inflammatory profile in the third day after hemorrhagic stroke is related to early mortality. On the other hand, CNS anti-inflammatory activity is related to survival.

Conclusions: Iron and heme are triggers of inflammatory response in the CNS after hemorrhagic stroke, and protection against hemoglobin and heme is lacking in the human brain. Inflammatory profile is associated with a poorer prognosis, while a local anti-inflammatory response seems to have a protective role.

Keywords: Iron, heme, cytokines, inflammatory response, hemopexine, haptoglobine.

Introduction

Inflammatory response has already been well documented after brain hemorrhage (1), and it is also clear that hemoglobin metabolism byproducts contribute to perpetuating brain injury. In experimental studies, iron was implicated in stimulating reactive oxygen species (ROS) formation and decreasing anti-oxidant defense (2). Iron was also found to prevent DNA repair (3), augment glutamate release (4), and amplify inflammatory response in the brain (5. In human studies, ferritin levels were correlated to poorer prognosis in ICH patients (6).

Free heme has been related to increased mortality in sepsis (7) and to the severe systemic manifestations of malaria (8). Free heme can also stimulate a pro-oxidant reaction (9), and augment inflammatory reaction through directly stimulating toll-like receptors (TLR) -4 (10). Neurons were found to be more sensitive to heme toxic effects than astrocytes, which contribute to perpetuate brain injury (11).

Haptoglobin (Hp) and hemopexin (Hx) are plasma proteins that are synthesized by the liver, and their functions are to bind free hemoglobin and heme, respectively, that have been released during intravascular hemolysis and to remove them from circulation. Some evidence suggests that Hp and Hx are involved in protecting the brain against injury after intracranial bleeding. Hypohaptoglobinemic and hemopexin-knockout mice present more neurological deficit and with reduced striatal cell viability after ICH, respectively (12), (13). However, in human data is scarce.

Despite ample experimental evidence, the role of iron and heme in the pathophysiology of brain injury after hemorrhagic stroke and of hemopexine and haptoglobine as potential protective mechanisms in humans is not fully understood. In this study, we aim to evaluate the role of blood metabolism products in the pathophysiology of brain injury after brain hemorrhage. We also strive to clarify the CNS mechanisms of

protection against iron and heme-induced damage and the relationship between inflammatory and blood metabolism parameters and early mortality.

Materials and Methods

Study Design and Population

Approval for the study was obtained from the local ethics committees of all participating hospitals, and in all cases informed consent was obtained from the patient or a surrogate. Fifteen patients with CT-documented ICH or SAH admitted to the neurocritical care units at Hospital Copa D'Or, Hospital Quinta D'Or and Hospital das Clínicas de Niterói (Rio de Janeiro, Brazil) were included. Eligibility criteria were spontaneous ICH or SAH with intraventricular hemorrhage (IVH) and external ventricular device (EVD) insertion within 24 hours after onset of symptoms. Exclusion criteria were age < 18 years, pregnancy and patients who are expected to survive less than 48 hours after admission. Demographic information was recorded on admission and severity of illness was assessed by calculating the Simplified Acute Physiology Score (SAPS) II, Glasgow Coma Scale, Hunt-Hess and Fisher scales for SAH and hematoma volume for ICH patients. Clinical information (heart rate, blood pressure, intracranial pressure, cerebral perfusion pressure) and laboratory results (blood count, eletrolytes, liver and kidney function parameters) were recorded sequentially. The primary outcome was 7-day mortality.

Blood and cerebrospinal fluid (CSF) were collected on the first 24, 48 and 72 hours after ICU admission. Blood was collected from an arterial line or a peripheral vein and CSF was collected from the EVD. Blood and CSF samples were assayed for cytokines, iron, heme, hemopexine, haptoglobine, S100-β and enolase concentrations. We also measured CRP-t, d-dimer, fibrinogen, PT and PTT in blood and in the CSF, cytometry, glucose and protein concentration.

Blood and CSF were put on ice and plasma and CSF supernatant were collected by centrifugation at 800 g for 15 min at 4°C, aliquoted and stored at -70°C until analysis. A multiplex cytokine kit (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL- 10, IL-12, IL-13, IL-17, IFN-γ, granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein-1 (MIP-1) and tumour necrosis factor [TNF]-α) was used according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Only the cytokines recovered in more than 70% of samples were analyzed (14).

Iron was measured by colorimetric assay as described by Carter (15). Iron is simultaneously released from protein and reduced by hydrochloric-thioglycolic acid. The ferrous dialysate reacts with buffered ferrozene, a monosodium salt of 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid), at a controlled pH and is then measured colorimetrically at 560nm. Total heme levels were measured using a chromogenic assay (GenWay Biotech, San Diego, CA) which utilizes peroxidase activity in the presence of heme to provide the conversion of a colorless probe to a strongly colored (λ = 570 nm) compound. Trace amounts of heme can be quantified in the 5-160pg (10-250 fmol) range.

Hemopexin (Hx), haptoglobin (Hp), enolase and S-100β concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (LifeSciences, Newberg, OR). In this assay, the Hx, Hp, enolase and S-100β present in samples reacts with their respective antibodies, which have been adsorbed to the surface of polystyrene microtitre wells. Then, these antibodies conjugated with horseradish peroxidase (HRP) are added. The enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) and measured at 450nm.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.0 for Mac (GraphPad Software, San Diego, CA, USA). Numeric variables are expressed as median (interquartile range) and were assessed using Mann-Whitney U-test and Kruskal-Wallis test. Dichotomous variables were analyzed using $\chi 2$ and Fisher's exact test (with Yates correction as indicated). Spearman analysis was employed to detect correlations among continuous variables.

Results

Patients Characteristics

Fifteen patients were included in this study, 6 patients (40%) died in the first 7 days after ICU admission. All of them were in mechanical ventilation on admission on the ICU and 11 (73.3%) were using vasoactive amines (table 1).

Concentrations of Iron, Heme, Haptoglobine and Hemopexine in Plasma and CSF

We measured the concentration of iron, heme, hemopexine and haptoglobine in the plasmatic and CSF compartments throughout the first three days after hemorrhagic stroke. Interestingly, not only CSF hemopexine and haptoglobine levels are almost undetectable and significantly lower than in plasma during the first three days after the event, but also their concentration does not increase during the early phase of hemorrhagic stroke. This finding suggests that these protection mechanisms against hemoglobin and heme within the brain parenchyma are lacking (table 2).

We identified that there was a decrease in plasmatic concentration of iron 48h after hemorrhagic stroke, which remained stable 72h after the ictus (243.4 x 74.85 x 94.4 mg/dl;

p=0.02). Concentration of heme, hemopexine and haptoglobine during the first three days after the event remained stable (table 3).

When we compared plasmatic and CSF concentrations of iron, heme, Hx and Hp, we found out that plasmatic iron concentration was significantly higher than CSF concentration 24h and 72 hours after hemorrhagic stroke. There was no difference between plasmatic and CSF concentrations of heme.

Relationship between iron and heme and plasmatic and CSF cytokines

We analyzed the correlation between iron and heme and cytokines concentration. There was a moderate negative correlation between plasmatic levels of iron 24 hours after the event and plasmatic IP-10 concentration 72 hours after hemorrhagic stroke (r= -0.67; p= 0.025). Interestingly, there was a strong correlation between CSF concentration of iron 48 hours after the ictus and CSF IP-10 levels 72 hours after the event (r=0.97; p=0.03).

Regarding heme, there was a strong correlation between CSF levels of heme in the first 24 hours after the ictus and MIP-1b concentration 48 hours after hemorrhagic stroke (r=0.76; p=0.01). CSF heme concentration in the first 48 hours after the event is also negatively correlated to CSF MCP-1 levels 72 hours after the ictus (r=-0.82; p=0.03). These data combined suggests that iron and heme may have a role in triggering inflammatory response within human brain after a hemorrhagic event. There was no correlation between iron and heme concentrations and other cytokines throughout the study period.

Concentration of Brain Injury Biomarkers in the Plasma and CSF

Regarding the kinetics of brain injury biomarkers, there was a steady increase in plasmatic concentration of enolase during the first three days after hemorrhagic stroke (2.65 x 4.85 x 38.06 mg/dl; p= 0.02). In parallel, CSF concentration of enolase progressively

decreased in the first 72 hours after the ictus (16.42 x 4.24 x 2.82; p=0.03). These results suggest that there is a preferential neuronal death over astrocyte with subsequent antigen spillover from the CSF into the blood.

Surprisingly, there was no change regarding S100-B kinetics, either in the plasmatic compartment or in the CSF.

Determinants of Early Mortality after Hemorrhagic Stroke

When we compared survivors with non-survivors 7 days after hemorrhagic stroke, we found out that, in the first 48 hours after the ictus, plasmatic iron and heme concentrations are higher in non-survivors than in survivors (496.04 x 58.5 mg/dl; p=0.05 for iron and 624.3 x 584.7 nM; p=0.04 for heme). This suggests that iron overload may contribute to brain injury and early mortality. There is no difference between survivors and non-survivors regarding hemopexine and haptoglobin concentration throughout the first three days after hemorrhagic stroke either in plasma or in the CSF.

Systemic and CNS inflammatory profile 72 hours after the event exhibits a consistent relationship with 7-day mortality. Within the brain parenchyma, CSF cytometry, lymphocyte and polymorphonuclear cell count are also significantly higher in non-survivors than in survivors 72 hours after hemorrhagic stroke (table 4).

We analyzed IL-1b, IL-2, IL-6, IL-8, GM-CSF, IP-10, MIP-1a, MIP-1b, IP-10 and RANTES in plasma. In the CSF, besides the cytokines analyzed in plasma, we also evaluated IL-4 and FGF. Three days after the ictus, plasmatic IL-6 and IL-8 are significantly higher in non-survivors than in survivors (1271 x 26.15 pg/ml; p= 0.04 for IL-6 and 134.8 x 3.83 pg/ml; p= 0.04 for IL-8).

On the other hand, local anti-inflammatory response seems to exert a protective role. In the CSF, IL-4 in the first 24 hours after hemorrhagic stroke is higher in survivors than in

non-survivors (34.98 x 0.001 pg/ml; p=0.04). There was no difference between survivors and non-survivors among the remaining cytokines either in plasma or in the CSF.

Surprisingly, there is no difference between survivors and non-survivors regarding enolase and S100-B concentrations either in plasma or in the CSF.

Discussion

In this study, we aimed to evaluate the role of hemoglobin metabolism byproducts and the protective mechanisms against hemoglobin and heme in the pathophysiology of brain injury after hemorrhagic stroke. We also attempted to find out whether iron, heme, hemopexine and haptoglobin are related to inflammatory response and 7-day mortality. Our main results were: 1) Defense against hemoglobin-derived injury is lacking – concentrations of hemopexine and haptoglobin in CSF are almost negligible comparing to plasmatic levels; 2) Iron and heme might be causally associated to inflammatory response, triggering CSF IP-10 and MIP-1b release and systemic iron overload is correlated to poorer prognosis; 3) Plasmatic and CSF inflammatory profile at 72 hours after the event are related to early mortality while local anti-inflammatory response might have a protective role after hemorrhagic stroke and SAH.

In this study, we found out that CSF haptoglobin and hemopexine concentrations are almost negligible comparing to plasma, and they do not increase during the first three days after the event. Although haptoglobin levels have already been related to lower mortality in septic patients (16), in the human brain, this protection seems to be lacking. This data cast doubt on the amount of Hb and heme scavenging that takes place in the human brain. It is also in agreement with Galea and colleagues report, who found out that most Hb was not bound to Hp, suggesting that the CD163-Hb-Hp system is saturated and that the primary route for Hb clearance from the CNS is freely crossing the blood brain barrier

through a concentration gradient (17). Therefore, we can conclude the mechanisms against hemoglobin and heme toxicity are lacking in the human brain, making the CNS more vulnerable the toxic effects of the hemoglobin degradation products.

In our study, IP-10 and MCP-1 concentrations were strongly correlated to iron and heme levels, respectively. The temporal association between iron and heme and IP-10 and MCP-1 release might suggest a causal relationship. However, IP-10 and MCP-1 release may be induced by another element (like thrombin), while iron and heme are only markers of the extension of bleeding.

The burden of systemic inflammatory response as a factor of poor prognosis is well known in hemorrhagic stroke patients. Systemic inflammatory response syndrome is seen in up to one-third of patients with SAH and is related to extra-cerebral organ dysfunction and worse outcome (18). Its components, as fever and leukocytosis, are markers of increased mortality (19),(20). Its frequency parallels the severity of the cerebral insult, being more common and of greater degree in higher grade radiographic and clinical SAH. The surge in ICP (21) and sympathetic nervous system activation (22) may both contribute to this strong relationship between severity of SAH and degree of SIRS. In our study, we found out that increased plasmatic concentrations of IL-6 and IL-8 in plasma were related to mortality. On the other hand, a local anti-inflammatory response, shown by increased CSF IL-4 levels, was a protective factor. The finding that CSF IL-4 was related to survival after hemorrhagic stroke is in synchrony with other studies which shown that anti-inflammatory activity has a neuroprotective role after brain injury (23), (24).

Our study has some limitations, like the small sample size and the mixed profile of patients with intracerebral bleeding and subarachnoid hemorrhage. The heterogeneity of cause of intracranial bleeding is a limiting factor, however, iron and heme have a key role in the pathophysiology of both diseases and this study provides preliminary evidence for a role of

iron and heme in triggering inflammatory response in the CNS and the lack of protection mechanisms against hemoglobin byproducts in human brain. Moreover, our study reinforces the notion of SIRS as an important factor in the outcome of hemorrhagic stroke patients. More extensive clinical studies of these biomarkers will be required to define their mechanistic and prognostic roles after hemorrhagic stroke.

Conflicts of Interest

The authors declare no conflicts of interest

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Table 1 – Patients characteristics

Characteristic	All Patients (n=15)
Age (years)[a]	59 (55-65)
Male Gender (%)	6 (40%)
Glasgow Coma Scale at admission[a]	7 (6-9)
Subarachnoid Hemorrhage	10 (66.6%)
Hemorrhagic Stroke (%)	5 (33.3%)
SAPS II[a]	43 (32-53)
Mechanical Ventilation at Admission (%)	15 (100%)
Shock at admission (%)	11 (73.3%)
7-day mortality (%)	6 (40%)
Hospital mortality	11 (73.3%)

Unless otherwise stated, values are expressed as number or percentage (%). [a] Median (interquartile range)

Table 2 – Comparison between plasma and CSF iron, heme, hemopexine and haptoglobine concentrations

	24h		48h		72h	
	Plasma	CSF	Plasma	CSF	Plasma	CSF
Iron (mg/dl)	243.4 (137.8-459.1)	50.93 (34.01-73.62)*	93.19 (58.5-254.1)	37.76 (32.92-170.2)	157.7 (80.71-506.4)	54.99 (43.57-72.26)*
Heme (nM)	628 (587-1125)	599.9 (591.9-643.8)	604.7 (583.4-633.2)	613.5 (591.7-745.9)	630.7 (594.8-658.8)	682.4 (639.6-1093)
Hemopexine (mg/dl)	46.11 (25.02-78.47)	0.95 (0-8.0)*	50.45 (17.16-113.8)	0 (0- 2.82)*	30.27 (15.46-65.37)	0.07 (0-4.74)*
Haptoglobin (mg/dl)	72.4 (42.9-156.3)	0.595 (0-4.898)*	109.3 (43.52-245.5)	0.865 (0-5.853)*	93.72 (59.18-205.5)	1.275 (0-6.175)*

Values are expressed by median (interquartile range).
*p< 0.05

Table 3 – Plasmatic and CSF iron, heme, hemopexine and haptoglobine kinetics during the first three days after hemorrhagic stroke

	24h	**48h**	**72h**
Plasma			
Iron (mg/dl)	243,4 (137,8-459,1)	74,85 (53,04-244,9)*	94,4 (3,67-167,3)
Heme (nM)	628 (587-1125)	604,7(583,4-633,2)	630,7 (594,8-658,8)
Hemopexine (mg/dl)	46,11 (25,02-78,47)	50,45 (17,16-113,8)	30,26 (15,46-65,37)
Haptoglobin (mg/dl)	72,4 (42,9-156,3)	109,3 (43,52-245,5)	97,32 (59,18-205,5)
CSF			
Iron (mg/dl)	50,93 (34,01 – 73,62)	37,76 (32,92-170,2)	54,99 (43,57-72,26)
Heme (nM)	599,9 (591,9-643,8)	613,5 (591,7-745,9)	682,4 (639,6-1093)
Hemopexine (mg/dl)	0,95 (0-8,0)	0 (0-2,82)	0,07 (0- 4,74)
Haptoglobin (mg/dl)	0,59 (0-4,89)	0,86 (0-5,85)	1,27 (0- 6,17)

Values are expressed by median (interquartile range). *p< 0.05

Table 4 – Relationship between inflammatory profile three days after hemorrhagic stroke and 7-day mortality

	Survivors (n=9)	**Non-Survivors (n=6)**
Plasma		
IL-6 (pg/dl)	26.15 (0.001 - 109.8)	1271 (250.7 - 4180)*
IL-8 (pg/dl)	3.83 (0.001 - 17.64)	134.8 (19.16-4062)*
CSF		
Cytometry (cells/mm3)	6 (5-9,25)	237 (140-1078)*
Red Blood Cell (cells/mm3)	13250 (3815-18406,25)	17685 (9619,5-34652,5)
PMN (cells/mm3)	0 (0-0)	58 (18,75-680,25)*
Lymphocytes (cells/mm3)	5 (3,5-6,5)	179 (121,25-397,75)*
Glucose (mg/dl)	69 (57,75-77,25)	100 (36,5-106)
Protein (mg/dl)	58,55 (44,47-92,2)	54(38,75-64,75)

Values are expressed by median (interquartile range). *$p<0.05$

ANEXO 4

Outras produções científicas durante o período.

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Cortisol levels and adrenal response in severe community-acquired pneumonia: A systematic review of the literature☆,☆☆,★,★★

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Keywords:
Adrenal failure;
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Corticosteroids;
Cortisol;
Sepsis

Abstract

Objectives: Our aim was to review the literature on the prevalence and impact of critical-illness related corticosteroid insufficiency (CIRCI) on the outcomes of patients with severe community-acquired pneumonia (CAP).

Methods: We reviewed Cochrane, Medline, and CINAHL databases (through July 2008) to identify studies evaluating the adrenal function in severe CAP. Main data collected were prevalence of CIRCI and its mortality.

Results: We screened 152 articles and identified 7 valid studies. Evaluation of adrenal function varied, and most studies used baseline total cortisol levels. The prevalence of CIRCI in severe CAP ranged from 0% to 48%. Among 533 patients, 56 (10.7%) had cortisol levels of 10 μg/dL or less and 121 patients (21.2%) had cortisol levels of 15 μg/dL or less. In a raw analysis, there was no significant difference in mortality when patients with cortisol levels less than 10 μg/dL (8.6 vs 15.5%; $P = .55$) or less than 15 μg/dL (12.4 vs 16%; $P = .38$) were compared with those with cortisol above these levels. In the meta-analysis, relative risk for mortality were 0.81 (confidence interval, 0.39-1.7; $P = .59$; $\chi^2 = 1.04$)

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for cortisol levels less than 10 μg/dL and relative risk was 0.67 (confidence interval, 0.4-1.14; $P = .84$; $\chi^2 = 1.4$) for cortisol levels less than 15 μg/dL.
Conclusions: A significant proportion of patients with severe CAP fulfilled criteria for CIRCI. However, CIRCI does not seem to affect the outcomes. Noteworthy, the presence of elevated cortisol levels is associated with increased mortality and may be useful as a prognostic marker in patients with severe CAP.

1. Introduction

Community-acquired pneumonia (CAP) is associated with significant morbidity and mortality and is the most common cause of death from infectious diseases in critically ill patients [1]. Patients with severe CAP often require intensive care unit (ICU) admission, and despite major advances in supportive care, an exceedingly high mortality rate is observed [2]. A recent study evaluating factors associated with early death in patients with CAP reinforces the classical concept that some deaths are not only dependent on antibiotic efficacy but also on other factors, especially inadequate host response [3]. The hypothalamic-pituitary-adrenal axis plays a major role in the regulation of the host's response to infection [4], and a strong association between elevated cortisol levels and severity of illness and the risk of death have been demonstrated [5-7]. Moreover, the presence of an inadequate adrenal response or adrenal dysfunction or, as more recently defined, critical illness-related corticosteroid insufficiency (CIRCI) may also be helpful in identifying patients with severe infections at high risk of death [5,8-10].

Complex changes in the endocrine system have been described in critical illness [11]. Severe infections and the immune host response to microorganisms are frequently implicated in the pathogenesis of adrenal response present in critically ill patients. Clinical and experimental data have demonstrated that pro and antiinflammatory mediators lead to decreased production and delivery of cortisol, overcome local tissue regulation of cortisone/cortisol ratio, and induce down-regulation of glucocorticoids receptors [12]. Thus, it can be easily noticed that the adrenal response is a complex phenomenon in critical illness and its diagnosis can be misleading. Moreover, its epidemiology and impact on the outcomes of patients with severe CAP remains to be established.

In the present article, we reviewed the medical literature, identified, and analyzed studies that evaluated the adrenal function in patients with severe CAP. We describe the frequency of CIRCI and whether it plays a significant role on the outcomes of patients with severe CAP.

2. Methods

2.1. Search strategy, study selection, data collection, and analysis

We performed a systematic search of Medline, Cochrane database, and CINAHL (from 1966 to July 2008) to identify full-text English language publications that evaluated the adrenal function in adult hospitalized with severe CAP. Inclusion criteria were established a priori. Major MESH search terms included community-acquired infections, pneumonia, adrenal insufficiency, adrenal failure, cortisol, corticosteroids, and glucocorticoids. Additional published reports were identified through a manual search of citations from retrieved articles. Only original peer-reviewed studies evaluating the adrenal function in adult patients with CAP were selected and analyzed. The abstracts of all articles were used to confirm our target population, and the corresponding full-text articles were reviewed for the presence of data evaluating the adrenal function of adult nonimmunocompromised patients with CAP. Two investigators (JIFS and CRS) independently identified the eligible literature. Predefined variables were collected, including year of publication; study design (prospective/retrospective, cohort/clinical trial); number of patients included; and hospital mortality and length of stay, oxygenation, frequency of septic shock, mechanical ventilation, and pneumonia severity stratification. Additional unpublished data were obtained by electronic mail from most authors. Any inconsistencies between the 2 investigators (JIFS and CRS) in interpretation of data were resolved by consensus. Standard descriptive statistics were applied to describe and compare the populations.

For evaluated homogeneity of studies, using Q Cochran test and I^2, the measure of effect was relative risk calculated using Mantel-Haenszel approach. All meta-analytic procedures were performed using R software version 2.10.1 and the package r meta version 2.16. Statistical analyses were carried out with the open source statistical language and environment R 2.9.0 [R Foundation for Statistical Computing, www.r-project.org].)

3. Results

The initial literature search yielded 152 articles, and 145 studies were excluded based on their titles and abstracts. The reasons for exclusion are shown in Fig. 1. Eventually, we found and analyzed 7 studies that evaluated the adrenal function of patients with CAP.

3.1. Description of studies and patient's characteristics

Different design and patient selection were observed in most studies. Overall, 533 patients were enrolled in 7

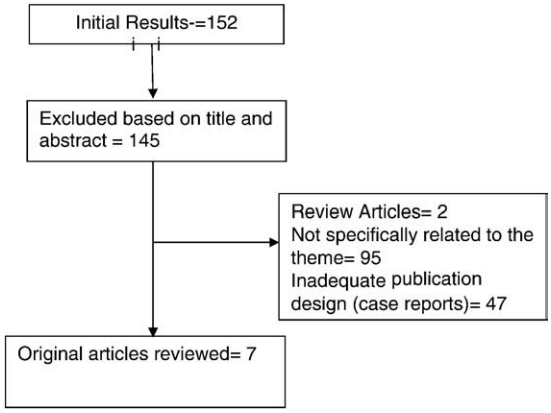

Fig. 1 Flow diagram of studies selected and reasons for exclusion.

studies that evaluated the adrenal function in patients with CAP (Table 1).

Overall, the studies evaluated a heterogeneous population of patients with CAP, ranging from mild CAP to those presenting with septic shock and respiratory failure (Table 1). Only 4 studies evaluated exclusively patients with severe CAP requiring ICU admission [8,13,17,20]. Feldman et al [13] included only critically ill patients with CAP, median Simplified Acute Physiology Score was 11.5 and ICU mortality was 33%. In the retrospective study performed by Salluh et al [20], 65% of the patients (n = 26) met the criteria for severe pneumonia according to the British Thoracic Society guidelines. These patients were severely ill as indicated by high Acute Physiology And Chronic Health Evaluation (APACHE) II scores (median, 16; 12-19; interquartile range, 25%-75%). In addition, a significant proportion of patients (70%; n = 28) received mechanical ventilation and were admitted in septic shock (47.5%; n = 19). The ICU and hospital mortality rates were 22.5% and 32.5%, respectively. Brivet et al [17] evaluated 38 patients, and 71% (n = 27) were diagnosed as severe CAP according to the American Thoracic Society criteria. Hospital mortality was 31.5%, and 27 patients (71%) needed mechanical ventilation. In the prospective study of Salluh et al [8], 72 CAP patients admitted to the ICU were evaluated. Patients were stratified with the CURB-65 [Confusion, Urea, Respiration, Blood pressure and Age 65 or more], median APACHE II score was 14 (11-17; interquartile range, 25%-75%), 27.8% of the patients received invasive mechanical ventilation, and 32% patients presented with septic shock. The ICU and in-hospital mortality were 13.8% and 16.7%, respectively. Among the studies that evaluated critically ill patients with CAP, median APACHE II ranged from 11 to 14, there was a high prevalence of mechanical ventilation (27.8%-71% of patients) and also a high prevalence of septic shock (32%-47.5% of patients). The ICU mortality varied from 13.8% to 22%, and hospital mortality ranged from 16.7% to 32.5%.

Christ-Crain et al [16] included 278 consecutive patients with suspected CAP admitted to the hospital, and 60% of patients (n = 167) were classified as severe CAP (pneumonia severity index [PSI] IV and V). Patients with PSI class IV and V had in-hospital mortality rates of 16% and 21%, respectively. Only 4 patients were hypotensive at presentation, and there is no available information about the use of mechanical ventilation or vasopressors or the need for ICU admission. Mikami et al [15] evaluated all patients admitted to the hospital with CAP but excluded those with septic shock and who needed mechanical ventilation or ICU admission. Seventeen patients (54.8%) were diagnosed as severe CAP (PSI classes IV and V), and only one patient died (3.2%). In the study conducted by Gotoh et al [18], all CAP patients admitted to the hospital were evaluated. Most patients (69%;

Table 1 Clinical studies evaluating the adrenal function in patients admitted with severe CAP

Reference	No. of patients	Patient category	Study design	End points
Feldman et al [13]	18	Severe CAP	Prospective single center cohort	Frequency of endocrine changes
Fine et al [14]	40	Severe CAP	Retrospective single center cohort	Evaluate cortisol levels
Mikami et al [15]	23	Moderate to severe CAP	Prospective single center cohort within an open-label prospective randomized controlled trial	Hospital length of stay, antimicrobial therapy duration, and time to stabilize vital signs
Christ-Crain et al [16]	278	CAP at emergency department presentation	Prospective cohort study	Correlation of adrenal function with survival
Brivet et al [17]	38	Severe CAP	Retrospective single center cohort	Correlation of cortisol levels with survival
Gotoh et al [18]	64	Moderate to severe CAP	Prospective single center cohort	Correlation of ACTH, cortisol and cortisol after cosyntropin-stimulation test with survival, and length of hospital stay
Salluh et al [8]	72	Severe CAP	Prospective single center cohort	Correlation of baseline cortisol levels and cortisol after cosyntropin stimulation with survival

n = 44) had severe CAP (PSI classes IV and V). There is no available information on septic shock, mechanical ventilation, or ICU admission, and 7 patients (10.9%) died during hospitalization. Among the studies that evaluated a non-ICU population of patients admitted with CAP, hospital mortality ranged from 3.2% to 21% of patients and was significantly lower than the critically ill population, as expected.

3.2. Diagnosis and prevalence of CIRCI

Diagnostic criteria of CIRCI have only recently been defined as a random total cortisol of 10 mg/dL or less or a δ serum cortisol of 9 μg/dL or less after adrenocorticotropic hormone (ACTH) administration of 250 μg [21]. As a result, several different criteria to address the adrenal function were used in each the selected studies. Among all, only total random cortisol levels were available for all patients. From 533 patients, 121 patients (21.2%) had baseline cortisol levels of 15 μg/dL or less and 56 (10.7%) had cortisol levels of 10 μg/dL or less. Christ-Crain et al [16] evaluated total and free cortisol levels in patients with CAP. In the whole study cohort, 54 patients (19.4%) had random total cortisol levels of 15 μg/dL or less and 30 patients (10.8%) had total cortisol levels of 10 μg/dL or less. Assessing only patients with PSI class IV and V (n = 147), 22 patients (14.9%) had total cortisol levels of 15 μg/dL or less, and 9 patients (6%) had total cortisol levels of 10 μg/dL or less [16]. Corticotropin stimulation test was not performed. Feldman et al [13] in an earlier study could not observe any case of CIRCI in patients with lobar pneumonia requiring ICU admission. Only baseline cortisol and ACTH levels were evaluated. The ACTH levels were nonsignificantly lower in nonsurvivors than in survivors, but values were not reported. Salluh et al [19] evaluated 40 patients with severe CAP. Random plasma cortisol levels were obtained, 5 patients (12.5%) had levels of 10 μg/dL or less and 19 patients (48%) had levels of 15 μg/dL or less. The ACTH levels or a corticotropin stimulation test were not obtained. Mikami et al [15] evaluated the adrenal function of 23 patients with CAP. One patient (4.3%) had baseline cortisol of 10 μg/dL or less, and 7 patients (40.3%) had levels of 15 μg/dL or less. A corticotropin (250 μg) stimulation test was performed, and the diagnostic criteria were fulfilled by 10 patients (43%). Critical illness-related corticosteroid insufficiency was not a predictive factor for either hospital length of stay or duration of intravenous antibiotic administration. No data on disease severity of this subgroup is available; only, there was no difference in disease severity or other clinical background between patients with or without CIRCI [15]. Gotoh et al [18] evaluated 64 patients hospitalized due to severe CAP and found that 2 patients (3%) had cortisol levels of 10 μg/dL or less and 12 patients (19%) had cortisol levels of 15 μg/dL or less. When corticotropin test was used as a diagnostic criterion of CIRCI, 13 patients (20%) fulfilled the diagnostic criterion [18]. Brivet et al [17] evaluated 38 severe CAP patients, 1 patient (2.7%) had cortisol levels of 10 μg/dL or less and 9 patients (25%) had cortisol levels of 15 μg/dL or less. A corticotropin test was not performed. Finally, Salluh et al [8] enrolled 72 patients with CAP admitted to the ICU. Seventeen (23.6%) had baseline cortisol levels of 10 μg/dL or less, and 20 patients (27.7%) had cortisol levels of 15 μg/dL or less. Corticotropin stimulation test was performed in all patients, and 13 (18%) were diagnosed as having CIRCI based on this criterion. Overall, the prevalence of CIRCI varied from 2.7% to 48% of patients, ranging from 2.7% to 23.6% when cortisol level of less than 10 μg/dL was used as CIRCI criteria and from 14.9% to 48% when cutoff was cortisol level of less than 15 μg/dL. Only 3 studies performed corticotropin stimulation test, and the prevalence of CIRCI according to these criteria were 18% and 43% [8,15,18] (Table 2).

3.3. Adrenal response and mortality

A total of 81 patients (15.2%) died during hospital stay. In a crude analysis, there was no significant difference in mortality between patients with CIRCI when compared to the non-CIRCI group (7/56 [8.6%] vs 74/477 [15.5%]; P =

Table 2 Prevalence of CIRCI and mortality in the clinical studies according to different CIRCI criteria

	No. of patients	Cortisol < 10 μg/dL	Cortisol ≥ 10 μg/dL	Cortisol < 15 μg/dL	Cortisol ≥ 15 μg/dL
Feldman	18	0	18 (33.3%)	0	18 (33.3%)
Salluh, 2006	40	5 (40%)	35 (22.8%)	19 (26.3%)	21 (38%)
Christ-Crain	278	30 (6.6%)	248 (11.7%)	54 (19.4%)	224 (12.5%)
Mikami	23	1 (0%)	22 (4.5%)	7 (0%)	16 (6.25%)
Brivet	38	1 (0%)	37 (32.4%)	9 (33.3%)	29 (31%)
Gotoh	64	2 (0%)	62 (11.3%)	12 (.8%)	52 (11.5%)
Salluh, 2008	72	17 (17.6%)	55 (16.4%)	20 (15%)	52 (17.3%)
Pooled studies	533	56 * (12.5%)	75 (15.7%)	121 ** (12.3%)	412 (18.3%)

In the study by Feldman et al [13], no patients presented low cortisol levels. Numbers in parenthesis represent mortality in the groups of patients with cortisol level of less than 10 and 10 μg/dL or greater and less than 15 and 15 μg/dL or greater.

* P = .55 (comparing mortality between cortisol < 10 μg/dL and ≥ 10 μg/dL).

** P = .38 (comparing mortality between cortisol < 15 μg/dL and ≥ 15μg/dL).

.55). When a baseline cortisol cutoff level of 15 μg/dL to define CIRCI was applied, again there was no difference in mortality (15/121 [12.4%] vs 66/412 [16.0%]; $P = .38$) (Table 3). When ICU vs non-ICU patients were compared, no significant difference in mortality was found in CIRCI patients when a cortisol cutoff level of less than 10 μg/dL was applied (5/23 [21.8%] vs 2/33 [6%]; $P = .11$). However, when a cortisol cutoff level of less than 15 μg/dL was used to define CIRCI, there was a significant difference in mortality between ICU vs non-ICU patients with adrenal dysfunction (11/48 [22.9%] vs 4/69 [5.8%]; $P = .009$) (Table 4). Only 3 studies have used corticotropin test to define CIRCI [8,15,18]. According to this criteria, when CIRCI vs non-CIRCI patients were compared, there was no significant difference in mortality (5/30 [16.6%] vs 15/121 [12.3%]; $P = .55$).

In the meta-analysis, when a cuttoff of basal cortisol level of less than 10 μg/dL was applied, we computed data for only 3 studies [8,16,20], due to the small number of CIRCI patients in the other studies. Relative risk for mortality was 0.81 (IC, 0.39-1.7; $P = .59$; $\chi^2 = 1.04$) (Fig. 2). When cortisol of less than 15 μg/dL was used as criteria, 5 studies were included [8,16-18,20]. Relative risk was 0.67 (IC, 0.4-1.14; $P = .84$; $\chi^2 = 1.4$) (Fig. 3).

4. Discussion

The current systematic review and meta-analysis comprehensively evaluates the role of cortisol levels and the diagnosis of CIRCI on mortality in patients with CAP. Analyzing the 7 selected studies, we could conclude that a diagnosis of CIRCI has no significant effect on mortality even when different cutoffs (baseline cortisol levels < 10 μg/dL or < 15 μg/dL) are considered. Our meta-analysis also has demonstrated no significant difference between CIRCI vs non-CIRCI patients. However, it suggests a possible association between high cortisol levels and mortality, which could make cortisol a useful biomarker for assessing prognosis in patients with severe CAP.

Regarding the impact of adrenal response on the outcomes, 2 of the evaluated studies thoroughly investigated and found an association between plasma cortisol and mortality [16,19]. These results are in accordance with those obtained from patients with severe sepsis [5-7]. Christ-Crain et al [16] observed that cortisol levels were directly associated with disease severity (as measured by the PSI score) and hospital mortality and concluded that cortisol levels are good predictors of severity and outcome in CAP. In this study, the prognostic accuracy of free cortisol for patients with CAP was not better than total cortisol. A total cortisol cutoff value of 34.8 μg/dL was superior to that of leukocyte count, C-reactive protein, and procalcitonin to predict death and improve the prognostic accuracy compared with the PSI alone [16]. Salluh et al [8] reported in a prospective study that there was no difference in ICU and hospital mortality between patients diagnosed with CIRCI and those who were not. Nonetheless, in this ICU population of patients with severe CAP, baseline total cortisol levels were significantly higher in nonsurvivors than in survivors. Also, baseline cortisol was the best predictor of death when compared with other laboratorial parameters (D-dimer and C-reactive protein) and scores (APACHE II, CURB-65, and SOFA). In this study, δ cortisol or postcorticotropin cortisol were not able to distinguish survivors from nonsurvivors. These data support the notion that although the presence of CIRCI is not associated with worse outcomes, elevated cortisol levels are associated with disease severity and in-hospital mortality.

Finally, it should be acknowledged that, despite the finding that low cortisol levels are not associated with worse outcomes in severe CAP, it does not mean that patients with severe CAP will not benefit from corticosteroids. Despite recent literature that challenges the role of adrenal function status in relation to the response to corticosteroids [21], there is also evidence of benefit of corticosteroids in patients with septic shock [22] and in selected patients with severe CAP [23]. Therefore, this issue is still a source of intense debate that should be evaluated in future clinical trials.

However, significant heterogeneity in study design and patient selection is observed among the studies and could

Table 3 Pooled analysis of mortality in patients with severe CAP according to different criteria of adrenal dysfunction

	Nonsurvivors	Survivors	Total	*P*
Cortisol < 10 μg/dL	7	49	56	.55 [a]
Cortisol > 10 μg/dL	74	403	477	
Cortisol < 15 μg/dL	15	106	121	.38 [b]
Cortisol > 15 μg/dL	66	346	412	

[a] For comparisons between survivors and nonsurvivors using cortisol level of less than 10 μg/mL as CIRCI criteria.
[b] For comparisons between survivors and nonsurvivors using cortisol level of less than 15 μg/mL as CIRCI criteria.

Table 4 Pooled analysis of mortality in ICU and non-ICU patients with severe CAP diagnosed with CIRCI according different criteria

	Nonsurvivors	Survivors	Total	*P*
Cortisol < 10 μg/dL				
ICU patients	5	18	23	.11 [a]
Non-ICU patients	2	31	33	
Cortisol < 15 μg/dL				
ICU patients	11	37	48	.009 [b]
Non-ICU patients	4	69	73	

[a] For comparisons between survivors and nonsurvivors using cortisol level of less than 10 μg/dL as CIRCI criteria.
[b] For comparisons between survivors and nonsurvivors using cortisol level of less than 15 μg/dL as CIRCI criteria.

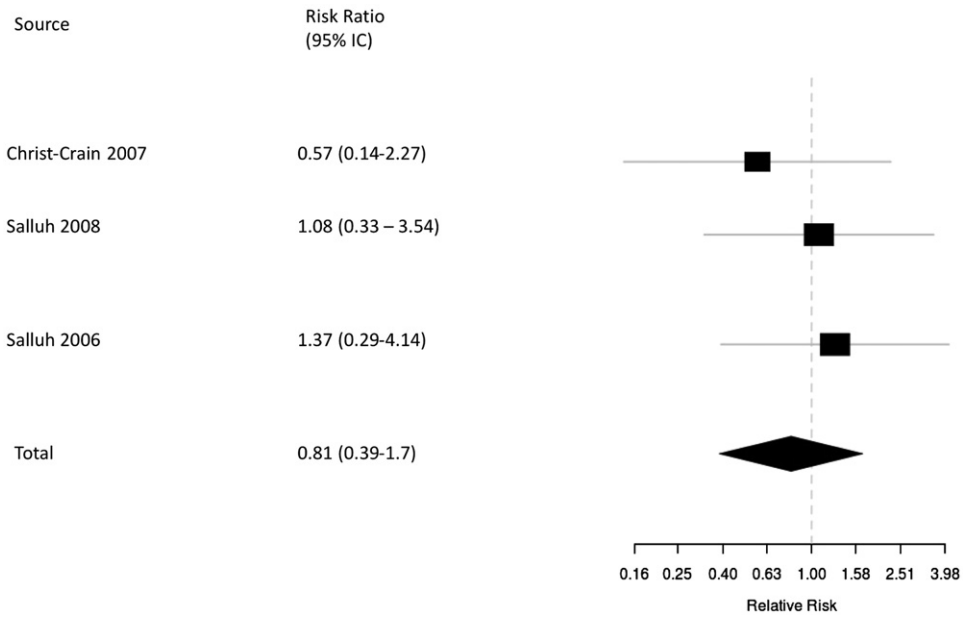

Fig. 2 Mortality based on basal cortisol levels of less than 10 μg/dL. CI indicates confidence interval. Size of the data markers indicates weight of the study.

explain the differences in the frequency of CIRCI and in its implications on clinical outcomes observed in the few studies currently available [5,7,10].

Selection bias is usually implicated as a plausible explanation for the results observed in clinical studies involving small patient population. Stratification for disease severity varied, and in only 2 studies [15,16], the same criteria was used. As disease severity varied among the studies, nonresponders to ACTH may have been underrepresented, and its influence on mortality may not been adequately recognized [24]. Unfortunately, adequate characterization and detailed data on subgroups as acute respiratory distress syndrome and septic shock were not available for all studies and could not be systematically evaluated. However, to overcome this, we obtained additional data from direct contact with the authors. Adrenal function was evaluated by many different methods including total cortisol [8,13,16], corticotropin test [15], and free cortisol [16], and different diagnostic criteria were applied by the investigators [5,25,26]. Currently, the diagnosis of

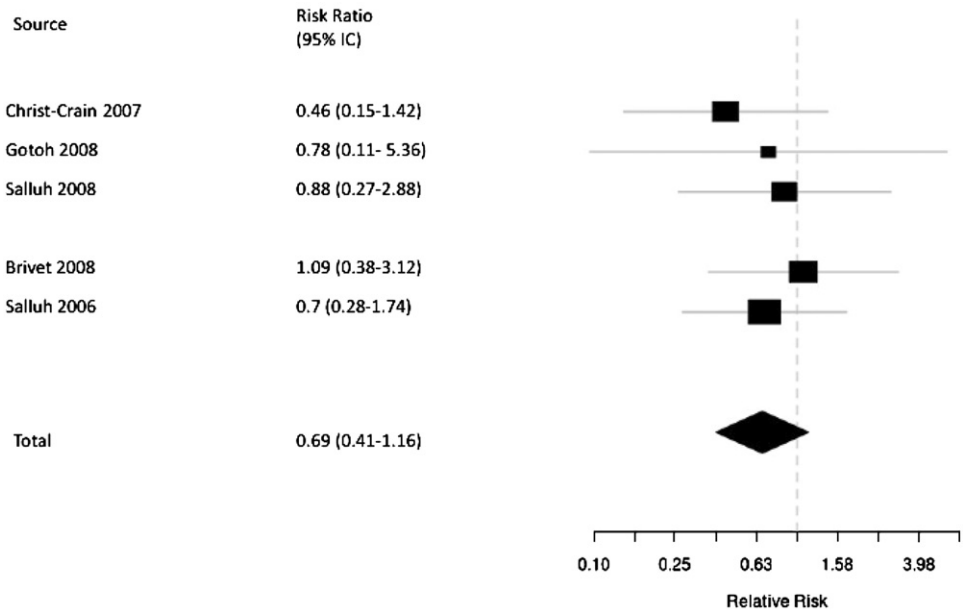

Fig. 3 Mortality based on basal cortisol levels of less than 15 μg/dL. CI indicates confidence interval. Size of the data markers indicates weight of the study.

adrenal dysfunction or CIRCI and its impact on the outcomes of severely ill patients are still matters of controversy [27,28], and the 250-μg ACTH infusion test is usually considered for the diagnosis of adrenal insufficiency [29]. In a landmark study, Rothwell et al [30] demonstrated that a failure to increase basal cortisol levels by greater than 9 μg/dL (nonresponse) after a 250-μg ACTH infusion was associated with increased mortality in patients with septic shock ($P < .001$). These findings were confirmed by a French multicenter study almost a decade later [5]. Despite these compelling data, several authors argue that the ACTH test is not appropriate for the diagnosis of CIRCI [8,17,31]. Hamrahian et al [32] have suggested the use of free cortisol concentrations to diagnose CIRCI. In addition, the question of what is an adequate baseline cortisol level continues to be debated, and several proposed baseline cortisol concentrations were evaluated. A baseline cortisol level of 15 μg/dL or less [33,34], or 10 μg/dL or less according to some authors, is considered sufficient to diagnose CIRCI [35]. To date, no single study evaluated exclusively patients with severe CAP by using simultaneously free and total cortisol, ACTH test, and methyrapone test.

5. Conclusions

In conclusion, the current evidence regarding the frequency and significance of adrenal dysfunction in patients with severe CAP is modest. Critical illness-related corticosteroid insufficiency is present in a variable number of patients with severe CAP (0%-48%) depending on the diagnostic methods and criteria applied in the different studies. Considering the present results, we cannot conclude that adrenal function tests are mandatory for the clinical management of patients admitted to the hospital with severe CAP. However, total cortisol levels may be useful as biomarkers for the assessment of disease severity and in-hospital outcomes.

After systematic review and meta-analysis, the authors, concerned about the quality of evidence available of effect the adrenal dysfunction in severe CAP, suggest that additional evidence based in prospective study with good sample size and well-defined end points is needed.

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Perceptions and practices regarding delirium, sedation and analgesia in critically ill patients: a narrative review

Percepções e práticas sobre delirium, sedação e analgesia em pacientes críticos: uma revisão narrativa

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ABSTRACT

A significant number of landmark studies have been published in the last decade that increase the current knowledge on sedation for critically ill patients. Therefore, many practices that were considered standard of care are now outdated. Oversedation has been shown to be hazardous, and light sedation and no-sedation protocols are associated with better patient outcomes. Delirium is increasingly recognized as a major form of acute brain dysfunction that is associated with higher mortality, longer duration of mechanical ventilation and longer lengths of stay in the intensive care unit and hospital. Despite all the available evidence, translating research into bedside care is a daunting task. International surveys have shown that practices such as sedation interruption and titration are performed only in the minority of cases. Implementing best practices is a major challenge that must also be addressed in the new guidelines. In this review, we summarize the findings of sedation and delirium research over the last years. We also discuss the gap between evidence and clinical practice and highlight ways to implement best practices at the bedside.

Keywords: Sedation; Delirium; Benzodiazepines; Propofol; Analgesics, opioid; Dexmedetomidine; Critical illness

INTRODUCTION

Sedation is commonly used in the intensive care unit (ICU), mainly in mechanically ventilated patients, to promote comfort, facilitate patient-ventilator interaction, and prevent self-harm.[1] In addition, deep sedation is often employed to reduce anxiety and promote amnesia in mechanically ventilated patients. Additionally, deep sedation allows healthcare practitioners to provide patient care in the ICU. However, the unrestricted administration of sedatives is frequently associated with oversedation,[2] which has been shown to increase the duration of mechanical ventilation and the lengths of ICU and hospital stays.[2-4] Over the past years, several studies were published that questioned the notion of deep sedation as standard of care.[2,3,5,6] Oversedation is associated with prolonged mechanical ventilation, longer ICU length of stay (LOS), increased delirium rates and increased mortality.[7,8]

Delirium is a frequent and severe form of acute brain dysfunction and is a major source of concern in critical care. Studies over the past

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ten years have clearly demonstrated an association between delirium and increased mortality, duration of mechanical ventilation, and hospital LOS.[9] Moreover, benzodiazepines, which were the most frequently used sedative drugs in ICU patients, were also associated with transitioning to delirium.[10] Despite the substantial evidence, these results have not been applied to clinical practice.[11-13] Surveys performed in different countries have shown conflicting results between ICU physicians' and nurses' perceptions of care and actual bedside practice.[14,15] In the present article, we present a narrative, non-systematic review to discuss the main advances in sedation research over the past 10 years and their impact on the care of critically ill patients.

Sedation guidelines

In 2002, the Society of Critical Care Medicine published its Guidelines for Sedation and Analgesia in Critical Care Adults.[16] These guidelines established a sedation goal that should be regularly reassessed for the individual patient with the systematic use of a validated sedation scale. Regarding the use of sedatives, the guidelines recommended benzodiazepines, namely lorazepam, as the first-line drug. It was recommended that midazolam be used for acutely agitated patients but only for short durations (48-72 hours). After this period, lorazepam was recommended for continuous or intermittent intravenous sedation. Propofol was suggested for neurosurgical patients or in other situations where rapid awakening was desirable. Dexmedetomidine was only briefly mentioned, and no recommendation for its use could be made at that time due to the lack of major studies in critically ill patients. Incorporating the findings of Kress et al.[2] and Kollef et al.,[7] these guidelines also recommended titration of the sedative dose to achieve an individual goal or a daily awakening strategy and the use of a sedation protocol.

Because most of the literature examining delirium in ICU patients is recent,[17] delirium is only briefly discussed in the 2002 sedation guidelines, which only emphasizes the need for routine assessment of delirium and the use of haloperidol as the drug of choice for delirium treatment.

Randomized controlled trials to decrease sedative exposure

Since the 2002 Guidelines for Sedation and Analgesia were published, sedation research has grown substantially, as seen in the graphic showing the exponential growth in PubMed citations over the last decade (Figure 1);[17] some pivotal studies are highlighted in figure 2. In 2000, Kress et al. showed that daily interruption of sedation reduced the duration of mechanical ventilation (4.9 versus 7.3 days; p=0.004) and also reduced ICU LOS (6.4 versus 9.9 days; p=0.02).[2] The impressive findings of this single-center study led to recommendations for "daily awakening" in the 2002 Guidelines.[17] Subsequently, Girard et al. performed a confirmatory multicenter study that compared daily awakening paired with a spontaneous breathing trial with the standard sedation care paired with a spontaneous breathing trial. Patients who underwent the intervention had decreased ICU (ICU LOS 9.1 versus 12.9 days; p=0.01) and hospital (hospital LOS 14.9 versus 19.2 days; p=0.04) lengths of stay.[5] There were more self-extubation events in the intervention group; however, the rates of reintubation were comparable. Interestingly, the intervention group showed improved 1-year survival (HR 0.68, 95% CI 0.50 to 0.92; p=0.01), representing a number needed to treat (NNT) of 7. This study certainly represents a major achievement over any contemporary critical care intervention trial. Recent data demonstrate that patients managed with protocolized sedation strategies do not benefit from the addition of daily sedation interruption because their durations of MV and ICU stays were unchanged.[18]

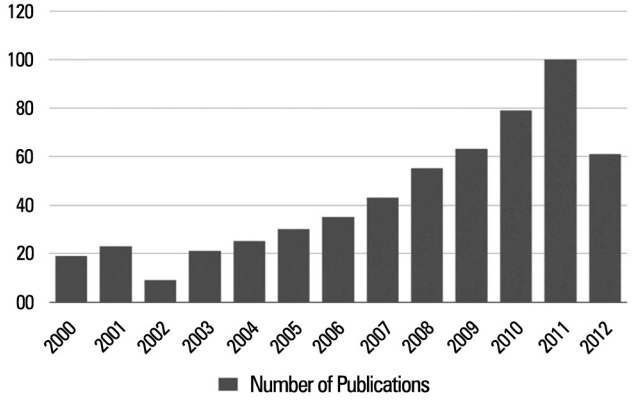

Figure 1 - Sedation and delirium research over the past 12 years.

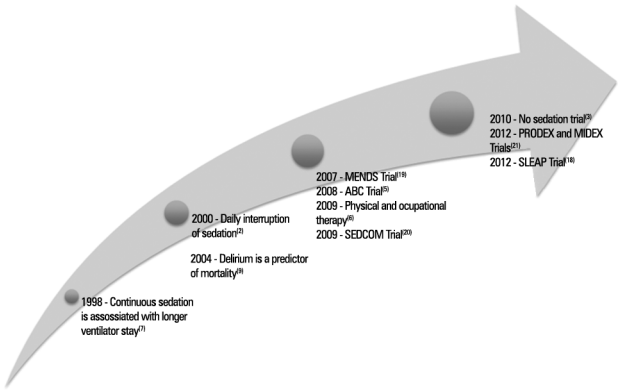

Figure 2 - Major sedation and delirium studies.

More recently, physical and occupational therapy coupled with daily interruption of sedation was compared with the use of daily interruption of sedation alone. Patients in the physical therapy group were more likely to return to an independent status at hospital discharge (59% versus 35%, p=0.02; odds ratio 2.7 [95% CI 1.2-6.1]), had a shorter duration of delirium (2.0 days, IQR 0.0-6.0 versus 4.0 days, 2.0-8.0; p=0.02), and had more ventilator-free days (23.5 versus 21.1 days, p=0.05).[6]

Subsequently, Strom et al. evaluated the impact of a "no-sedation protocol" on the outcomes of mechanically ventilated patients. Patients were randomized to no-sedation (only morphine bolus as needed) or sedation (propofol for 48 hours, midazolam thereafter plus morphine bolus as needed) with daily awakening. In this single-center study, the intervention group had significantly more days without ventilation (mean difference, 4.2 days; 95% CI 0.3-8.1; p=0.019) as well as shorter stays in the intensive care unit (HR 1.86, 95% CI 1.05-3.23; p=0.031) and hospital (3.57, 1.52-9.09; p=0.004).[3] Interestingly, the control group was already managed with the best evidence-based care to date, which makes the results even more impressive. Regarding the controversies surrounding daily suspension of sedation and protocolized sedation, it was previously shown that daily sedation interruption does not add to protocolized sedation strategies, insofar as the sedation goals are met.[18]

Substantial progress has also been made regarding the occurrence of delirium and sedation choice. Since the early 2000s, delirium was recognized as prevalent and was associated with worse outcomes in ICU patients. In a landmark prospective cohort study, Ely et al. demonstrated that delirium was independently associated with 6-month mortality in mechanically ventilated patients (adjusted HR, 3.2; 95% confidence interval [CI], 1.4-7.7; p=0.008).[9] Since then, studies have demonstrated the association of different sedative drugs with the occurrence and severity of delirium.[9,10] Benzodiazepine exposure was associated with delirium transitioning in several studies. Pandharipande et al. demonstrated that lorazepam was an independent risk factor for daily transition to delirium (odds ratio, 1.2; 95% confidence interval, 1.1-1.4; p=0.003) in a dose-dependent manner.[10] However, similar results were not obtained for propofol or fentanyl.[9] Other studies corroborate this finding. In a 1-day point-prevalence multicenter study involving 104 ICUs in 11 countries, Salluh et al. found that delirium was associated with increased mortality and ICU LOS and that, among sedatives, midazolam was associated with a diagnosis of delirium.[8]

In 2007, a randomized controlled trial termed the MENDS study tested the hypothesis that a benzodiazepine-sparing sedation strategy could reduce the occurrence of acute brain dysfunction in mechanically ventilated patients.[19] Patients in the dexmedetomidine group spent more time at a targeted level of sedation (80% versus 67%; p=0.04) and had more days without delirium or coma (7.0 versus 3.0 days; p=0.01), mainly due to reduced incidence of coma (63% versus 92%; p<0.001). Two years later, the SEDCOM study compared the efficacy and safety of dexmedetomidine versus midazolam in medical/surgical patients who were expected to remain on mechanical ventilation for more than 24 hours.[20] The secondary end-points were prevalence and duration of delirium. Dexmedetomidine was comparable to midazolam for achieving a targeted sedation; however, patients in the dexmedetomidine group had less delirium (54% versus 76.6%, p<0.001) and less time to extubation (3.7 versus 5.6 days, p=0.01), although the ICU length of stay was similar in both groups. Interestingly, the main outcomes examined in MIDEX and PRODEX (non-inferiority trials comparing dexmedetomidine to midazolam and propofol) were the proportion of time in light-to-moderate sedation (RASS scores between 0 and -3) and the duration of mechanical ventilation.[21] Dexmedetomidine was comparable to midazolam and propofol for achieving light-to-moderate long-term sedation; however, it reduced the length of mechanical ventilation compared with midazolam (123 versus 164 hours; p=0.03) but not when compared with propofol

(97 versus 118 hours; p=0.24). In both studies, there was no difference in the number of patients who needed to be sedated due to delirium, although the incidence of delirium was evaluated only once at 48 hours after stopping the sedative drugs.[21] Taken together, the results of these studies suggest that benzodiazepines are associated with increased risk of acute brain dysfunction, and interventions that reduce benzodiazepine use can improve relevant clinical outcomes in mechanically ventilated critically ill patients.

Progress in sedation research over the last decade was reflected in the 2013 guidelines for sedation, analgesia and delirium endorsed by the Society of Critical Care Medicine, as shown in table 1.

Table 1 - Major differences between the 2002 and 2013 sedation guidelines

Topics	2002	2013
Number of recommendations	28	33
Pain assessment	Numeric rating scale (NRS)	Behavioral pain scale (BPS) and the Critical care pain observation tool (CPTO)
Sedation goal	A sedation goal should be implemented	Light sedation is the goal for the majority of patients
Sedation assessment	Validated sedation scale (SAS, MAAS or VICS)	Most validated sedation scale (RASS or SAS)
Sedation strategy	Use of sedation protocols	Either daily interruption of sedation or light target level of sedation
Sedation selection	Lorazepam is the drug of choice for most patients	Preference for non-benzodiazepine sedatives
Delirium risk factor	None	Benzodiazepine use
Delirium prevention	None	Early mobilization is recommended

Current use of sedation: perception and practices of healthcare practitioners

Several surveys were published in the last decade that focused on the practice of sedation worldwide[12,22,23] (Table 2). Although the majority of these studies report self-perception, some audits were performed as well, and they reveal startling differences between physicians' statements and actual clinical practice.

In 2001, Soliman et al. published the largest sedation survey in Europe, which included 647 ICU physicians distributed in 16 countries. These authors reported that morphine and fentanyl were equally employed for opioid-based analgesia (33% each) followed by sufentanil (23%). The most frequently used sedative drug was midazolam (63%) followed by propofol (35%), and lorazepam was infrequently used (<0.5%).[23] In 2009, Tanios et al. performed a survey of 904 critical care practitioners (60% physicians, 14% nurses and 12% pharmacists) in the United States. According to the current guidelines, the most frequently used sedative agents were lorazepam and midazolam, and propofol was selected as the first-choice sedative by only 13-26% of responders. Morphine was primarily used for analgesia.[22] Patel et al. surveyed 1384 health care practitioners (70% physicians, 23% nurses and 1.6% respiratory therapists) in North America about sedation. In that study, benzodiazepines (84%) and propofol (81%) were the most commonly used sedative agents.[24]

In contrast, an Australian-New Zealand survey performed in 2010 with critical care physicians and nurses showed that midazolam and propofol were equally used (50% each) as the sedation drug of choice, and again, morphine was used as the first choice for analgesia (67%) followed by fentanyl (13%).[15] These findings may be indicative of a cultural difference regarding the approach to sedation.

Regarding the adherence to daily interruption of sedation, the results also varied significantly across countries. However, adherence was generally low, varying from 14% in Malaysia[25] and 15% in Nordic countries[26] to 31% in Denmark[27] and 34% in Germany.[28] Patel et al. reported that the majority of respondents (76%) had a written policy on spontaneous awakening trials. However, less than half of the health care practitioners evaluated (44%, 446/1019) performed spontaneous awakening trials on more than half of the ICU days.[24] Recently, Australia-New Zealand[15] and UK surveys[29] revealed higher levels of self-reported sedation interruption (62 and 78%, respectively). A study comparing sedation interruption in ICUs in Germany showed that from 2002 to 2006, there was a 34% increase (23 to 45% of ICUs) in the implementation of sedation interruption.[30] Unfortunately, despite evidence of the dangers of continuous sedation and oversedation, the practice of sedation interruption has not yet been implemented in most ICUs, creating a large evidence-to-practice gap.

The use of written sedation protocols is strongly encouraged as a way to promote a consistent approach to individually targeted sedation, but it also varies in different countries. Martin et al. reported a 21%

Table 2 - Summary of surveys dealing with sedation that have been published in the last decade

Study	Year	Site of study	Number of respondents	Healthcare worker evaluated (%)	Daily interruption of sedation (%)	Sedation protocol (%)	Sedation scale (%)	Sedation goal (%)
Murdoch et al.[31]	2000	England	255	Physicians	Not reported	Yes (27)	Yes (67)	Not reported
Soliman et al.[23]	2001	Europe	647	Physicians	Not reported	Not reported	Yes (43)	Not reported
Guldbrand et al.[26]	2004	Nordic countries	88	Not reported	Yes (15)	Yes (41)	Yes (53)	Not reported
Martin et al.[28]	2006	Germany	305	Physicians	Not reported	Not reported	Not reported	Not reported
Egerod et al.[27]	2006	Denmark	82	Physicians (47.5), nurses (52.5)	Not reported	Yes (physicians-23/ nurses-9)	Yes (nurses-30/ physicians-44)	Not reported
Martin et al.[30]	2007	Germany	220	Physicians	Yes (34% increase from 2002 to 2006)	Yes (46)	Yes (46)	Not reported
Ahmad et al.[25]	2007	Malaysia	37	Physicians	Yes (14)	Yes (35)	Yes (35)	Not reported
Mehta et al.[14]	2007	Canada	88	Nurses	Not reported	Not reported	Not reported	Not reported
Reschreiter et al.[29]	2008	UK	192	Not reported	Yes (78)	Yes (80)	Yes (88.1)	Not reported
Patel et al.[24]	2009	United States	1,384	Physicians (70), nurses (23.2), respiratory therapists (1.6)	Yes (76)	Yes (71)	Yes (88)	Not reported
Tanios et al.[22]	2009	United States	904	Physicians (60%), nurses (14), pharmacists (12)	Yes (40)	Yes (64)	Not reported	Not reported
Salluh et al.[32]	2009	Brazil	1,015	Physicians	Yes (31.7)	Yes (52.7)	Yes (88.3)	Yes (37.2)

frequency of use in German ICUs,[28] whereas other authors showed a 27% use in the UK[31] and a 33% use in Denmark.[27] Patel et al. reported that 29% of respondents did not have a written sedation protocol.[24] Earlier surveys reported an increasing trend in using sedation protocols, ranging from 52% in Germany[28] to 80% in the UK.[31] The Ramsay scale appeared to be the most commonly used scale for sedation assessment in the various surveys.[28-31]

In Brazil, a study published in 2009 with 1015 critical care physicians found that midazolam and fentanyl were the most frequently used sedative agents (97.8 and 91.5%, respectively) followed by propofol (55%).[32] Only 52.7% of respondents reported using a sedation protocol in their ICUs. Approximately 62.8% of physicians reported not discussing sedation targets in daily rounds, and 68.3% did not practice sedation interruption at all.

Changing sedation practices and critical care culture: a major challenge

Implementing change in clinical routines is complex and labor intensive. Sedation audits in the ICU reveal a different reality from that which is reported by surveys. In an audit performed in 1,381 adult patients admitted to 44 ICUs in France, Payen et al. reported that midazolam was the most commonly used sedative agent (70%) followed by propofol (20%).[11] Opioid-based analgesia was implemented mainly with sufentanil (40%) and fentanyl (35%). A large proportion of patients underwent deep sedation (40-50%), and regular assessment for sedation and analgesia was significantly lower than the use of sedatives and opioids. None of the ICUs performed sedation interruption, and procedural analgesia was infrequently used (less than 25% of patients). In a Canadian study that included 52 ICUs, Burry et al. reported that sedative and analgesia interruptions were performed only 20% and 9% of the time, respectively. These authors also found that only 8% of patients underwent sedative dose adjustment based on the use of a validated sedation scale.[33]

The impact of clinical trials on current practice is low overall, and there are many plausible explanations. Gaps in the dissemination of knowledge, skepticism about the cost-effectiveness of the practice (cost being perceived as financial resources and effort), doubts about personnel and equipment support and applicability to an

individual setting are frequently cited as major barriers to implementing new practices.[34] Tanios et al. reported that the three most common reasons that prevented multidisciplinary teams from adopting sedation scales were no physician order (35%), lack of nursing support (11%), and fear of oversedation (7%). The main reasons that prevented teams from adopting daily interruption of sedation were lack of nurse acceptance (22%), concern about risk of patient-initiated device removal (19%), and inducement of either respiratory compromise (26%) or patient discomfort (13%).[22] In another study, O'Connor and Bucknall found that nurses were more likely to believe that daily interruption of sedation would increase their workload.[15] In the same study, when asked about other factors that could influence sedation management, physicians and nurses alike cited level of experience and education and support from staff. Other factors cited by nurses were staffing level and pressure for beds, whereas physicians most often cited unit culture ("a quiet patient is a good patient"). Cost is also an important issue in clinical decision-making, being cited by 52% of ICU physicians in the UK[27] and 64% in Maghreb.[35]

New guidelines for sedation and analgesia in critical care will incorporate these changes; however, guideline publishing is not enough to translate good evidence into good bedside practice.[34] Carey et al. suggested that guidelines should focus not only on the best evidence available but also on planning strategies for its best implementations and pilot studies for evaluating implementation plans.[36] Gesme and Wiseman have also advocated for a leadership role and an organizational culture that support change to help implement the best practices.[37] However, studies have shown that even complex quality improvement strategies may be successfully implemented in the ICU setting.[38,39]

CONCLUSION

Despite all the available evidence, best sedation practices are still heterogeneous and insufficiently implemented worldwide. It is imperative to address the obvious gap between research and practice. More data are needed to help establish the best implementation strategies and help provide the best care to all patients admitted in the intensive care unit.

RESUMO

Durante a última década, foi publicado um número significativo de estudos fundamentais que aumentaram o conhecimento atual sobre a sedação em pacientes criticamente enfermos. Desse modo, muitas das práticas até então consideradas como padrão de cuidado são hoje obsoletas. Foi demonstrado que a sedação excessiva é perigosa, e que protocolos com sedação leve ou sem sedação se associaram a melhores desfechos dos pacientes. O *delirium* vem sendo cada vez mais reconhecido como uma forma importante de disfunção cerebral associada com mortalidade mais alta, maior duração da ventilação mecânica e maior permanência na unidade de terapia intensiva e no hospital. Apesar de todas as evidências disponíveis, a tradução da pesquisa para o cuidado ao pé do leito é uma tarefa hercúlea. Foi demonstrado, por levantamentos internacionais, que práticas como interrupção e titulação da sedação só são realizadas em uma minoria dos casos. O estabelecimento das melhores práticas é um tremendo desafio que deve também ser contemplado nas novas diretrizes. Nesta revisão, resumimos os achados de estudos a respeito de sedação e *delirium* nos anos recentes e discutimos a distância entre a evidência e a prática clínica, assim como as formas de estabelecer as melhores práticas ao pé do leito.

Descritores: Sedação; Delírio; Benzoadepinas; Propofol; Analgésicos opióides; Dexmedetomidina; Estado terminal

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Implementing sedation protocols: closing the evidence-practice gap

Implementando protocolos de sedação: aproximando a diferença entre evidência e prática

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Sedation and analgesia are frequently used in the critical care unit. Pain has already been described as the "fifth vital sign," and most people describe experiencing pain as a source of great stress during an intensive care unit (ICU) stay.[1,2] Sedation can be used to ease discomfort, to facilitate adaptation to mechanical ventilation, and to prevent self-harm.[3] However, despite its humanitarian intentions, over-sedation is associated with prolonged mechanical ventilation, increased delirium rates, longer ICU lengths of stay (LOS), and increased mortality.[4,5]

In recent decades, many studies have addressed the risks of over-sedation.[6] Kress et al. were the first to demonstrate that a protocol of daily awakening led to a reduced duration of mechanical ventilation and of ICU LOS.[7] Subsequently, Girard et al. performed a trial comparing daily awakening plus spontaneous breathing trials with standard sedation practices plus spontaneous breathing trials and showed that the intervention group had an improved 1-year mortality, with an impressive NNT of 7.[8] More recently, a "no-sedation, analgesia-based" trial also showed more ventilator-free days and reduced ICU and hospital LOS.[9]

Despite all the impressive evidence available, there is a wide variation among sedation surveys worldwide. Self-reported adherence to daily interruption of sedation varies from 14% in Malaysia[10] to 78% in the UK.[11] In North America, Patel et al. showed that only 44% of the respondents performed sedation interruption on more than half of the ICU days, and 29% did not have a written sedation protocol.[12] The use of a sedation protocol also varies among countries, ranging from 33% in Denmark[13] to 80% in the UK.[14] In Brazil, a recent survey showed that only 52.7% of the respondents use a sedation protocol, and 68.3% of physicians do not practice sedation interruption at all.[15]

Why there is such a wide evidence-practice gap? There are many possible explanations, such as the lack of personnel or equipment support, concern about risk of patient-initiated device removal, and fear of patient discomfort and increase in workload.[16] In this context, the trial presented in this edition of the journal by Bugedo et al. clarifies much.[17] The authors performed a nationwide, multicenter study in 13 ICUs evaluating an analgesia-based, goal-directed, nurse-driven sedation protocol. They showed that after an educational effort, the proportion of patients in deep sedation or coma could be reduced from 55.2% to 44% with no increase in agitation events. This paper shows us that the implementation of sedation protocols is feasible, although it requires a persistent educational effort and the participation of all of the staff working in the ICU.

Conflicts of interest: Former speaker from Hospira.

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The Impact of Acute Brain Dysfunction in the Outcomes of Mechanically Ventilated Cancer Patients

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Abstract

Introduction: Delirium and coma are a frequent source of morbidity for ICU patients. Several factors are associated with the prognosis of mechanically ventilated (MV) cancer patients, but no studies evaluated delirium and coma (acute brain dysfunction). The present study evaluated the frequency and impact of acute brain dysfunction on mortality.

Methods: The study was performed at National Cancer Institute, Rio de Janeiro, Brazil. We prospectively enrolled patients ventilated >48 h with a diagnosis of cancer. Acute brain dysfunction was assessed during the first 14 days of ICU using RASS/CAM-ICU. Patients were followed until hospital discharge. Univariate and multivariable analysis were performed to evaluate factors associated with hospital mortality.

Results: 170 patients were included. 73% had solid tumors, age 65 [53–72 (median, IQR 25%–75%)] years. SAPS II score was 54[46–63] points and SOFA score was (7 [6–9]) points. Median duration of MV was 13 (6–21) days and ICU stay was 14 (7.5–22) days. ICU mortality was 54% and hospital mortality was 66%. Acute brain dysfunction was diagnosed in 161 patients (95%). Survivors had more delirium/coma-free days [4(1,5–6) vs 1(0–2), $p<0.001$]. In multivariable analysis the number of days of delirium/coma-free days were associated with better outcomes as they were independent predictors of lower hospital mortality [0.771 (0.681 to 0.873), $p<0.001$].

Conclusions: Acute brain dysfunction in MV cancer patients is frequent and independently associated with increased hospital mortality. Future studies should investigate means of preventing or mitigating acute brain dysfunction as they may have a significant impact on clinical outcomes.

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Introduction

Delirium is a common type of acute brain dysfunction in patients admitted to the intensive care unit (ICU) [1,2]. To date, several studies have demonstrated that delirium is associated with increased risk of mortality as well as increased hospital length of stay (LOS) and costs [1–3]. In addition, when high-risk populations are considered, such as the elderly and mechanically ventilated, delirium may occur in up to 80% of ICU patients [2]. The impact of delirium on relevant clinical outcomes is not restricted to the hospital setting as delirium is also an independent predictor of six-month mortality and long-term cognitive impairment [2,4,5]. However, most epidemiological data derives from general ICU patients and critically ill cancer patients have not been thoroughly evaluated. Cancer patients may present high risk for acute brain dysfunction as it is associated with several factors such as high burden of comorbidities, chronic exposure to opioids and sedatives, acute and chronic systemic inflammation among others. Currently up to 20% of all ICU patients have a diagnosis of cancer [6,7] and while predictors of in-hospital mortality and clinical outcomes are well described for this population [6,8–10] to the best of our knowledge none of the studies investigated the occurrence and impact of delirium and acute brain dysfunction in a systematic way. The aim of the present study was to evaluate the frequency of acute brain dysfunction and its impact on outcomes of mechanically ventilated cancer patients.

Patients and Methods

Design and setting

The present study is a prospective cohort study performed in the ICU of Instituto Nacional de Câncer (INCA), Rio de Janeiro, Brazil. The ICU is a fifteen-bed medical-surgical unit specialized

in the care of patients with cancer [8], with the exception of bone marrow transplant patients.

Briefly, during the study period (February 2010 to February 2012), every adult cancer patient (≥18 yrs) that required ICU admission was consecutively evaluated. Patients in complete remission >5 yrs, those ventilated for more than 24 h prior to ICU admission, patients ventilated for less than 48 h in the ICU and readmissions were not considered. Legal blindness and deafness and the inability to speak Portuguese as well as moribund patients (expected to die <24 h) were also excluded. The main outcome of interest was hospital mortality.

Definitions, Selection of Participants and Data Collection

Demographic, clinical and laboratory data were collected using standardized case report forms and included main diagnosis for ICU admission, the Simplified Acute Physiology Score (SAPS) II [11] the Sequential Organ Failure Assessment (SOFA) score [12], comorbidities, and cancer- and treatment-related data. Level of arousal was measured using the RASS score [13] rates a patient's level of agitation/sedation on a 10-point scale ranging from −5 (unarousable, not responsive to voice or physical stimulation) to +4 (combative). Coma was defined as a RASS score of minus 4 (responsive only to physical stimulus) or minus 5 (unresponsive to physical stimulus) of any cause as previously defined [14]. Delirium was diagnosed with the CAM-ICU [15]. The CAM-ICU was developed for use in critically ill, intubated patients and is a validated delirium detection tool with high sensitivity and specificity and high inter-rater reliability [16] that was validated in Portuguese by our group [17]. The CAM-ICU assesses four features of delirium: (1) acute onset or fluctuating course, (2) inattention, (3) disorganized thinking, and (4) altered level of consciousness. To be considered CAM-ICU positive, the subject must display features 1 and 2, and either 3 or 4. The CAM-ICU was applied every morning by two trained investigators (I.C.A and V.C.S-D) to every eligible patient during the first 14 days of ICU stay. The ICU and hospital mortality rates from any cause were also assessed.

This study was supported by institutional funds and did not interfere with clinical decisions related with patient care. The Ethics Committee of the Instituto Nacional de Câncer in Rio de Janeiro approved the study (Number 144/2009) and the need for informed consent was waived.

Data processing and Statistical Analysis

Data entry was performed by the investigators (I.C.A, V.C.S-D) and consistency was assessed with a rechecking procedure of a random sample of patients. Data were screened in detail by two investigators (J.I.F.S., I.C.T) for missing information, implausible and outlying values.

Standard descriptive statistics were used. Continuous variables were reported as median [25%–75% interquartile range (IQR)]. Univariate analysis was used to identify factors associated with hospital mortality. Two-tailed *P*-values <0.05 were considered statistically significant. Univariate and multivariable logistic regression were used to identify factors associated with hospital mortality. Variables yielding *P*-values below 0.2 by univariate analysis were entered into a forward multivariable logistic regression analysis. Clinically relevant variables such as: sepsis,

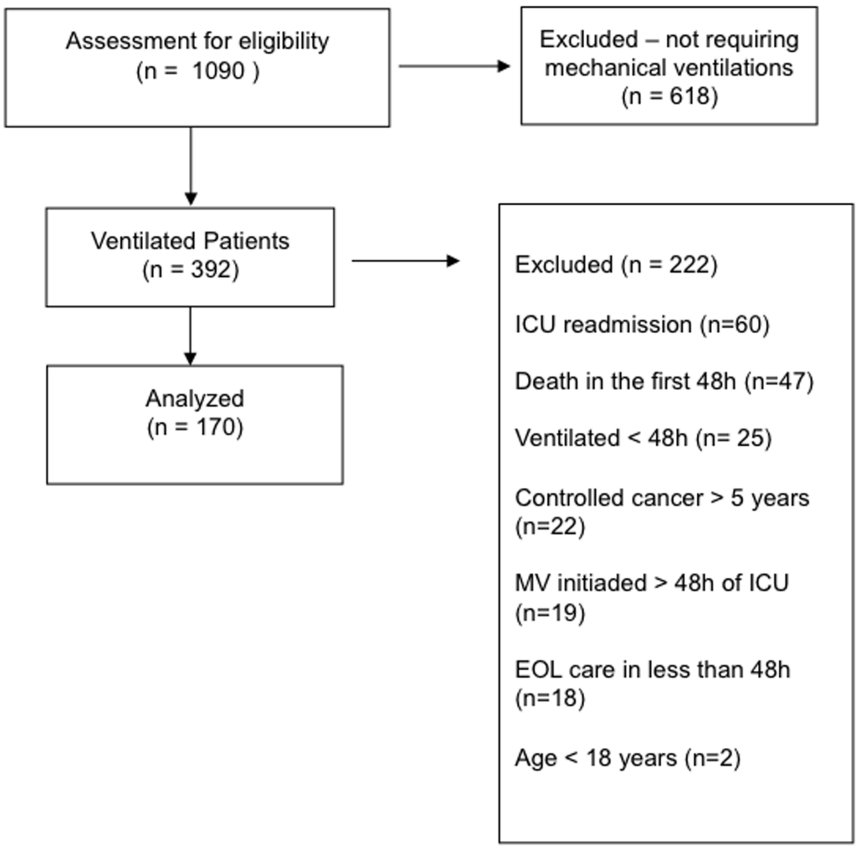

Figure 1. Study Flow Diagram.
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use of sedatives, chemotherapy, cancer status, age and co-morbidities were forced into the model. Multivariable analysis results were summarized by estimating odds ratios (OR) and respective 95% confidence intervals (CI). Possible interactions were tested. The area under the receiver-operating characteristic curve was used to assess the models' discrimination. The SPSS 13.0 software package (Chicago, Illinois, USA) and Prism 3.0 (Graphpad, USA) were used for statistical analysis.

Results

Characteristics of the study population

After the initial screening of 1090 consecutive ICU admissions, a total of 170 patients that fulfilled inclusion criteria were enrolled in the study (Figure 1). The main characteristics including cancer-related variables of the study population are depicted in Table 1. Overall, ICU and hospital mortality were 54.7% and 66.4%%, respectively. One hundred and thirteen patients (66.4%) were admitted to the ICU due to a medical condition while emergency and elective surgery represented 25.2% and 8.2% of cases, respectively. At ICU admission, sepsis was the most frequent diagnosis (n = 108, 63.5%).

Diagnosis of acute brain dysfunction: Associated Characteristics and Outcomes

After excluding patients deeply sedated and unarousable with RASS deeper than −3 during the entire study period, delirium was evaluated with the CAM-ICU in 126 patients (74% of the entire eligible patient population). Daily interruption of sedation [18] was a part of routine ICU care and performed according to local protocol based on Kress et al [18].

Overall, delirium was diagnosed by the CAM-ICU in 92.8% of patients (n = 117/126) of the included arousable patients. Detailed comparisons between patients with and without a diagnosis of acute brain dysfunction (ABD) are also depicted on table 1.

Regarding hospital mortality, a comparison was performed between survivors and non-survivors (including the whole cohort). As expected, survivors presented lower severity of illness as expressed by the SAPS II scores (50 [43–60] vs 56(47–63), p = 0.011). Additionally, ventilator free-days and delirium-coma free days were higher in survivors. The results regarding the comparison of other variables are shown in Table 2.

Variables selected in the univariate analysis (those with p-values<0.2 and others with clinical interest regardless of p-value such as: age, charlson index, cancer type and status) were entered in multivariable analysis. In addition to the SAPSII, only acute brain dysfunction as well as delirium/coma free-days were selected in the final models and independently associated with hospital

Table 1. Demographic and clinical variables of patients according to the presence of acute brain dysfunction.

Variables	All Patients (n = 170)	Acute Brain Dysfunction (n = 161)	No acute brain dysfunction (n = 9)	*P*-value*
Age (years)	63(53–72)	62(53–72)	64(50–68)	0.78
Male gender, n (%)	100(58.8%)	93(57.7%)	7(77.7%)	0.36
***Performance status* (3–4), n (%)**	34(20%)	33(20.4%)	1(11.1%)	0.68
Cancer status (recent diagnosis/relapse/progression), n (%)	161(94.7%)	152(94.4%)	9(100%)	0.99
Solid tumor, n (%)	125(73.5%)	118(73.2%)	7(77.7%)	0.99
Tumor extension (locally advanced/distant metastasis), n (%)	78(45.8%)	75(46.5%)	3(33.3%)	0.64
SAPS II score (points)	54(46–63)	54(45–63)	57(50–60)	0.76
Charlson comorbidity index (points)	2(2–3)	2(2–3)	3(2–4.5)	0.50
SOFA score (points)	7(6–9)	7(6–9)	6(4.5–7)	0.07
Type of admission				
Medical n (%)	113(66.4%)	107(66.4%)	6(66.6%)	0.99
Main reasons for ICU admission				
Sepsis, n (%)	108(63.5%)	105(65.2%)	3(33.3%)	0.08
Respiratory failure (excluding sepsis), n (%)	27(15.8%)	22(13.6%)	5(55.5%)	0.006
PaO2/FiO2 (points)	270(200–380)	270(200–380)	270(140–390)	0.60
Sedatives, n (%)	168(98.8%)	161(100%)	7(77.7%)	0.002
MV LOS (days)	13(6–21)	13(6.5–20)	15(5–28)	0.99
ICU LOS (days)	14(7.5–22)	14(7–22)	13(10–20)	0.94
Hospital LOS (days)	26(14–39)	26(13–39)	36(21–49)	0.13
ICU mortality, n (%)	93(54.7%)	90(55.9%)	3(33.3%)	0.34
Hospital mortality, n(%)	113(66.4%)	110(68.3%)	3(33.3%)	0.06
End of life care, n (%)	30(17.6%)	27(16.7%)	3(33.3%)	0.25

*For comparisons among patients with and without the diagnosis of acute brain dysfunction.
SAPS II - Simplified Acute Physiology Score II; SOFA - Sequential Organ Failure Assessment; ICU - intensive care unit; LOS –length of stay; Performance is status is defined according to the Eastern Cooperative Oncology Group (ECOG) scale.
Results expressed as median (25%–75% interquartile range) and number (%).
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Table 2. Comparison of Survivors and non-survivors.

Variables	Survivors (n = 57)	No Survivors (n = 113)	*P*-value
Age (years)	64(53–70.5)	62(53–73)	0.53
Male gender, n (%)	31(54.3%)	69(61%)	0.41
Performance status (3–4)	10(17.5%)	24(21.2%)	0.68
Cancer status (recent diagnosis/relapse/progression), n (%)	55(96.4%)	106(93.8%)	0.71
Solid tumor, n (%)	45(78.9%)	80(70.7%)	0.27
Tumor extension (locally advanced/distant metastasis), n (%)	30(52.6%)	48(42.4%)	0.25
SAPS II score (points)	50(43–60)	56(47–63)	0.0112
Charlson comorbidity index (points)	2(2–3)	2(2–3)	0.54
SOFA score (points)	7(5.5–9)	7(6–9)	0.60
Type of admission - Medical, n (%)	32(56.1%)	81(71.6%)	0.05
Sepsis, n (%)	37(64.9%)	71(62.8%)	0.86
P/F score (points)	280(190–380)	270(200–384)	0.85
Sedatives, n (%)	56 (98.2%)	112 (99.1%)	0.99
Delirium/Coma	51(89.4%)	110(97.3%)	0.06
Delirium/coma-free days	4(1,5–6)	1(0–2)	<0.0001
MV LOS (days)	9(6.5–18)	14(6–22)	0.29
Ventilator free days (days)	3(1–5.5)	0(0-0)	<0.0001
ICU LOS (days)	14.5(10–20.5)	13(6–23)	0.33
Hospital LOS (days)	26(25.5–53)	21(10–33)	<0.0001

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mortality (Table 3). As there was potential colinearity between the presence of acute brain dysfunction and coma-delirium free-days two models were fitted containing either the acute brain dysfunction or delirium-coma free-days. In multivariable analysis, acute brain dysfunction (OR = 5.00 [95% CI, 1,15–21.68], p = 0.03) and delirium-coma free-days (0.771 [0.681 to 0.873], p<0.001) were associated with increased hospital mortality.

We also analyzed mortality of two groups stratified by the median duration of (median = 1) of delirium/coma free-days and observed higher cumulative mortality (84.8 vs 46.1%, p = 0.001) in patients that presented more acute brain dysfunction (Figure 2). Data on the mortality stratified by 3 categories of duration of delirium/coma free days is also provided in Figure 3.

Understanding the evidence of a spectrum that encompasses delirium, coma plus delirium and coma, we analyzed separately those patients that were comatose though all the study period. As expected when the 44 patients with RASS deeper than −3 for the whole study period were compared to the remaining 126 patiens we observed that they had higher SOFA scores (8[6–10] vs 7[5–8], p = 0.07), less ventilator-free days (0[0-0] vs 1[0–1], p<0.01), increased ICU (93.1% vs 41.2%, p<0.0001) and hospital mortality (95.4% vs 56.3%, p<0.0001) as compared to arousable patients regardless of diagnosis of delirium.

Table 3. Multivariable analyses of factors associated with increased hospital mortality.

Variables	Coefficient	Odds-Ratio (95% CI)	*P*-value
Model containing the Delirium/Coma			
Delirium/Coma	1.610	5.00 (1,15–21.68)	0.03
SAPSII Score (points)	0.029	1.03 (1,002–1.059)	0.03
Surgical admission	−0.659	0,52(0.259 to 1.031)	0.06
Constant	−2.155		
Model containing the Delirium/Coma Free Days			
SAPSII Score (points)	0.032	1.032 (1.003 to 1.063)	0.028
Coma-Delirium Free Days	1.21	0.771 (0.681 to 0.873)	<0.001
Constant	−0.325		

Model containing the Delirium/Coma: Area under receiver operating characteristic curve = 0.67 (95% CI, 0.59 to 0.74).
Model containing the Delirium/Coma- Free Days: Area under receiver operating characteristic curve = 0.75 (95% CI, 0.68–0.81).
SAPSII - Simplified Acute Physiology Score II; CI – confidence interval.
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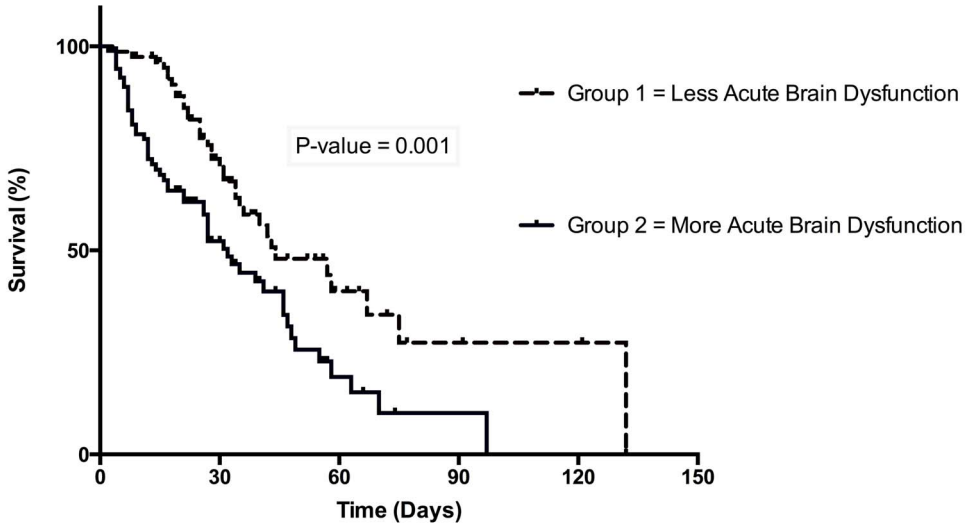

Study Day	0	15	30	60	90
Group 1	78	72	40	9	2
Group 2	92	54	30	5	2

Figure 2. Kaplan–Meier analysis depicting the impact of delirium and coma on hospital mortality. Group 1- Less acute brain dysfunction represents patients with delirium/coma free-days >1 day. Group 2- More acute brain dysfunction represents patients with delirium/coma free-days ≤1.
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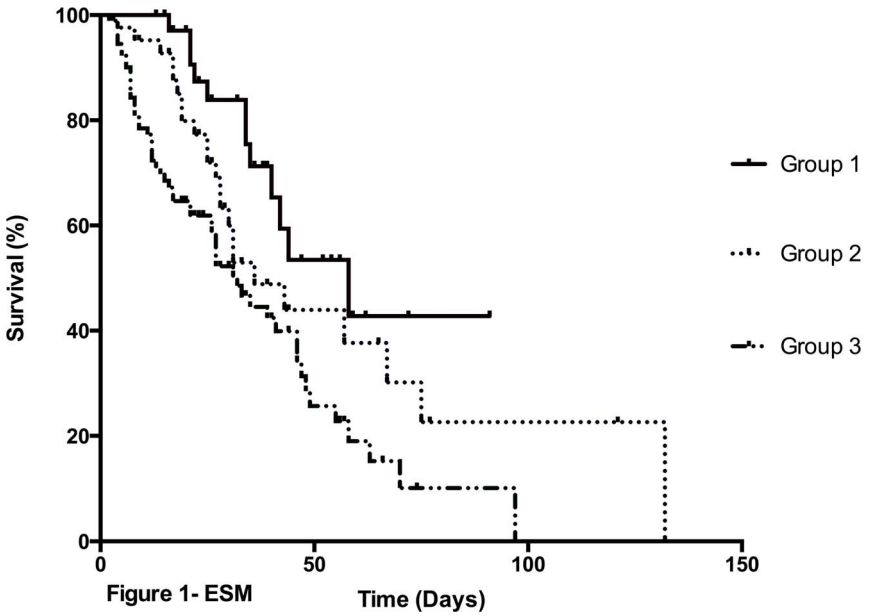

Figure 1- ESM

Kaplan–Meier analysis depicting the impact of delirium/coma on hospital mortality

Group 1 = Delirium/Coma Free-Days > 4

Group 2= Delirium/Coma Free-Days Between 2 and 4

Group3= Delirium/coma Free-Days less or equal to 1

Figure 3. Kaplan–Meier analysis depicting the impact of delirium/coma on hospital mortality.
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Discussion

In the present study, we evaluated a prospective cohort of mechanically ventilated patients with cancer patients and observed that the frequency of acute brain dysfunction is considerably high. Moreover, acute brain dysfunction is a major predictor of mortality for this population.

In the past decade, several studies increased the knowledge of factors associated with hospital mortality for critically ill patients with cancer [8,10,19,20]. These studies demonstrated that the severity of acute illness and organ dysfunctions [10] as well as patients' comorbid conditions and performance status were important determinants of short-term outcomes. The knowledge of these factors have been considered important to aid the bedside clinician to avoid forgoing intensive care for patients with a chance of survival and to improve resource allocation [10,21,22].

Global mortality rates observed in our population are exceedingly high, however are comparable to studies enrolling cancer patients with severe sepsis or those necessitating ventilatory support [6,9]. Although one recognizes the importance of knowing the classic predictors of mortality in critically ill cancer patients, it should be stressed that none of them are modifiable giving clinicians little room for interventions other than a well structured ICU triage procedure and discussions on EOL care. In this sense the information that acute brain dysfunction is frequent and associated with poor outcomes in this population may be useful to test the effectiveness of interventions and help improve the current mortality rates. Several studies have demonstrated that different pharmacologic and non-pharmacologic interventions may reduce the incidence of acute brain dysfunction in mechanically ventilated patients in general ICUs [14,23–25]

Several factors may help explain why patients with cancer present a high frequency of acute brain dysfunction such as chronic pain and opiod use, chronic sustained systemic inflammation, older age, high burden of comorbidities, use of steroids and terminal illness [26,27]. Studies evaluating non-ICU cancer patients requiring hospitalization have demonstrated that delirium occurs in up to 42% of patients [28,29]. In a recent study that evaluated patients submitted to esophageal resection delirium occurred in 50% of the patients in the post-operative period and associated with increased duration of mechanical ventilation and hospital stay [30]. However, data on critically ill cancer patients, especially the mechanically ventilated, are scarce.

The present study has some limitations. First it was a single-center study performed at a specialized center, however the patients' characteristics did not differ significantly from those in multicenter studies [6,7]. Also, the sample size although calculated based on the mean prevalence of delirium in mechanically ventilated in contemporaneous studies [14,23] ended up being limited and precluding subgroup analysis such as sepsis, sedative use and other relevant characteristics and risk factors and due to the unexpectedly high rate of acute brain dysfunction. Therefore it was underpowered for comparison among groups such as delirium and no-delirium. Additionally, delirium was evaluated only once a day and as it is a fluctuating syndrome some diagnoses may have been missed. However, due to the already elevated rates of acute brain dysfunction observed in our cohort we believe this impact would not be as important as if we were in a setting with lower overall rates. In addition, the fact of being performed in a specialized unit did not allow a "control group" with non-cancer patients. A study by Neufeld et al have demonstrated hat in non-critically ill hospitalized cancer patients, the CAM-ICU and ICDSC intensive care delirium screening tools are not adequately sensitive for use in routine clinical practice, although this could be a potential issue, the fact that our rates of acute brain dysfunction were very high diminishes the potential impact of such finding [31].

Also delirium subtype (a relevant clinical feature) was not evaluated. Also, we did not evaluate adherence to process of care measures that could impact in the frequency of delirium, although the unit has implemented sedation protocols as standard of care [32]. Aspects related to the cumulative dose and sedation depth over time were not registered. Therefore it was not possible to perform a comparison of patients stratified by the presence or absence of modifiable risk factors of delirium. And finally, no long-term follow-up was performed and therefore from present data we cannot draw conclusions on the impact of ABD on long-term cognitive function and quality of life of these patients. Importantly, as a cohort study, we demonstrated the association of acute brain dysfunction (a potentially modifiable predictor of outcome) and hospital mortality in mechanically ventilated cancer patients. However, a clinical trial is required to clearly demonstrate causal relation between interventions that reduce the frequency and duration of acute brain dysfunction will improve hospital survival in critically ill cancer patients.

Conclusions

In conclusion, acute brain dysfunction is present in most mechanically ventilated cancer patients and is independently associated with mortality. Strategies aiming at the reduction of the frequency, severity and duration of this condition should be implemented in this population and tested in a population of critically ill patients with cancer.

Author Contributions

Conceived and designed the experiments: JIFS ICTA CRS RB VCS MS FAB EWE. Analyzed the data: JIFS. Wrote the paper: JIFS MS FAB EWE. Contributed data management and patient inclusion: ICTA CRS RB VCS. Revised the manuscript: ICTA CRS RB VCS.

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