UNIVERSIDADE DE CRUZ ALTA
UNIVERSIDADE REGIONAL DO NOROESTE DO ESTADO DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM ATENÇÃO INTEGRAL À SAÚDE

PROTEÍNAS DE CHOQUE TÉRMICO NA PERDA AUDITIVA INDUZIDA PELO RUÍDO: BIOMARCADORES E ALVOS TERAPÊUTICOS

DISSERTAÇÃO DE MESTRADO

MARCOS SOARES

IJUÍ – RS, Brasil

2017

PROTEÍNAS DE CHOQUE TÉRMICO NA PERDA AUDITIVA INDUZIDA PELO RUÍDO: BIOMARCADORES E ALVOS TERAPÊUTICOS

Por

MARCOS SOARES

Dissertação apresentada ao Programa de Pós-Graduação em Atenção Integral à Saúde, da Universidade de Cruz Alta (UNICRUZ, RS), em associação ampla à Universidade Regional do Noroeste do Estado do Rio Grande do Sul (UNIJUI, RS), como requisito parcial para a obtenção do grau de **Mestre em Atenção Integral à Saúde**

Orientador: Prof. Dr. Thiago Gomes Heck

IJUÍ – RS, Brasil

2017

Catalogação na Publicação

S676p Soares, Marcos.
Proteínas do choque térmico na perda auditiva induzida pelo ruído: biomarcadores e alvos terapêuticos / Marcos Soares. – Ijuí, 2017.
86 f.: il. ; 30 cm.

Dissertação (mestrado) – Universidade Regional do Noroeste do Estado do Rio Grande do Sul (Campus Ijuí). Atenção Integral à Saúde.

"Orientador: Thiago Gomes Heck".

1. Ouvido – Distúrbios funcionais. 2. Perda auditiva – Indução por ruído. 3. Perda auditiva – Proteínas do choque térmico. I. Heck, Thiago Gomes. II. Título.

CDU: 616.28-008

Carla Inês Costa dos Santos
CRB10/973

UNIVERSIDADE DE CRUZ ALTA
UNIVERSIDADE REGIONAL DO NOROESTE DO ESTADO DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM ATENÇÃO INTEGRAL À SAÚDE

A Comissão Examinadora, abaixo assinada,
aprova a dissertação de Mestrado

PROTEÍNAS DE CHOQUE TÉRMICO NA PERDA AUDITIVA INDUZIDA PELO RUÍDO: BIOMARCADORES E ALVOS TERAPÊUTICOS

elaborada por

MARCOS SOARES

Como requisito parcial para obtenção do grau de
Mestre em Atenção Integral à Saúde

Prof. Dr. Thiago Gomes Heck
Orientador

COMISSÃO EXAMINADORA

Profa. Dra. Letícia Petersen Rosito (UFRGS)

Prof. Dr. Matias Nunes Frizzo (UNIJUI)

Profa. Dra. Mirna Stela Ludwig (UNIJUI)

Ijuí, 28 de abril de 2017

Dedico este trabalho à minha família, em especial a minha esposa Gabriela e ao meu filho Felipe que com muito apoio e carinho incentivaram para que eu chegasse até aqui. Amo vocês!

AGRADECIMENTOS

Agradeço a Deus por me dar a família linda que tenho e por ter colocado pessoas tão boas em meu caminho.

Aos meu pais por sempre acreditarem em mim e não medirem esforços para que eu pudesse sempre seguir em frente.

Aos meus irmãos Maurício, Marcelo e Mariel, que apesar da distância, sempre torcem por mim.

À minha amada esposa, Gabriela, que sempre me incentivou para seguir em frente, mesmo nos momentos adversos. Obrigado pela paciência e compreensão. Te amo!

Ao nosso filho Felipe, um presente de Deus, que veio alegrar ainda mais a minha vida.

Ao meu orientador, Thiago Gomes Heck, pela oportunidade, pelos ensinamentos, pela paciência e pela amizade que selamos. Obrigado!

À Mirna Stela Ludwig pelas puxadas de orelha, pelos ótimos conselhos científicos, pelos ensinamentos durante o mestrado, e, principalmente, por nossa amizade. Obrigado!

Ao Grupo de Pesquisa em Fisiologia - GpeF, em especial a Analu Bender dos Santos, pela ajuda durante a pesquisa, pelos

conhecimentos do mundo do laboratório e pela amizade; a Marlon Turcato, pela ajuda nos experimentos.

À fonoaudióloga Tainara Milbradt Weich pelo suporte durante os experimentos.

A todos os colegas do PPGAIS pelo companheirismo e troca de experiências.

A todos os professores do PPGAIS pelos ensinamentos.

Aos professores componentes da banca examinadora desta dissertação, pela disponibilidade em contribuir com este trabalho, em especial a Letícia Petersen Rosito, pelos ensinamentos em Otologia e por vir de tão longe para prestigiar a banca.

RESUMO

A perda auditiva é uma desordem sensorial prevalente nos seres humanos que afeta a capacidade individual de comunicação oral. Muitos fatores podem levar ao dano sensorioneural, principalmente ao órgão de Corti. Diante das situações de estresse no ouvido interno, proteínas de choque térmico de 70kDa (HSP70) são expressas. As HSP70 intracelulares (iHSP70) são descritas como tendo ações anti-inflamatórias, promovendo citoproteção. Por outro lado, no meio extracelular (eHSP70) estas proteínas apresentam funções pró-inflamatórias. Portanto, a busca por evidências de substâncias ou condições que são capazes de modificar as concentrações de HSP70 é um campo promissor para o desenvolvimento de estratégias de prevenção e tratamento de doenças do sistema auditivo. A expressão intracelular da isoforma induzível de 72 kDa (iHSP72) pode ser potenciada pela suplementação com dipeptídeo alanilglutamina (DIP). Analizamos se a perda auditiva induzida por ruído promove a alteração nas concentrações de iHSP72 e eHSP72 (HSP72 extracelular), e também verificamos se a suplementação com DIP pode modificar essas concentraçções e prevenir a perda auditiva. Ratos fêmeos Wistar (n=32), com 90 dias de idade, foram divididos aleatoriamente em grupos Controle (CON), expostos ao ruído (NIHL), tratados com alanilglutamina (DIP) e tratados com alanilglutamina e expostos ao ruído (DIP + NIHL). O potencial evocado auditivo de tronco cerebral foi avaliado antes e 14 dias após a exposição ao ruído (124 dB SPL durante 2 h). Cóclea, núcleo coclear e amostras de plasma foram recolhidos para medir iHSP72 e eHSP72 por kit ELISA de alta sensibilidade. A exposição ao ruído induziu um aumento no limiar auditivo, não evitado pelo tratamento DIP. Houve aumento dos níveis de iHSP72 e eHSP72 no grupo NIHL, que foi evitado pelo tratamento DIP. Também o índice-H (razão plasma/cóclea eHSP72/iHSP72) foi elevado no NIHL e suprimido pelo tratamento com DIP. Nossos dados indicam que o dano coclear induzido pela exposição ao ruído é acompanhado pela resposta local e sistêmica ao choque térmico. Além disso, a suplementação de alanilglutamina, com o protocolo testado, não diminuiu a perda auditiva induzida pelo ruído, mas reduziu os marcadores de estresse. Estes dados sugerem que o nível plasmático de proteína de choque térmico de 72 kDa pode ser usado como biomarcador da condição de estresse auditivo após a exposição ao ruído.

Palavras-chave: perda auditiva, resposta de choque térmico, iHSP70, eHSP70, exposição ao ruído, alanilglutamina, índice-H.

ABSTRACT

HEAT SHOCK PROTEINS IN NOISE INDUCED HEARING LOSS: NEW PATHWAYS AND THERAPEUTIC PERSPECTIVES

Hearing loss is a prevalent sensory disorder in humans impacting on individual ability to oral communication. Many factors can lead to sensorineural damage, mainly to the Corti organ. Faced with stress situations on inner ear, HSP70, an important cytoprotective protein, is expressed. Intracellular HSP70s (iHSP70) are described as having anti-inflammatory molecular actions, promoting cytoprotection through anti-apoptotic mechanisms, inhibiting gene expression and regulating cell cycle progression. On the other hand, in the extracellular *milieu* these proteins participate as pro-inflammatory signal. Therefore, evidences substances or conditions that are able to induce iHSP70 expression in cochlea is a promising field for development of strategies for prevention and treatment of diseases of auditory system. The iHSP72 expression can be potentiated by alanyl-glutamine dipeptide (DIP) supplementation. We argue whether noise induced hearing loss (NIHL) promotes both intracellular (iHSP72) and extracellular (eHSP72) Heat Shock Response (HSR) alteration and if DIP supplementation can modify HSR and prevent hearing loss. Female Wistar rats (n = 32), 90 days old, were randomly divided in Control (CON), Noise induced Hearing Loss (NIHL), treated with DIP (DIP) and NIHL plus DIP (DIP+NIHL). Auditory Brainstem Response was evaluated before and 14 days after noise exposure (124 dB SPL for 2 h). Cochlea, nuclear cochlear complex and plasma samples were collected to measure iHSP72 and eHSP72 by high sensitivity ELISA kit. The noise exposition induced an increase in auditory threshold, not prevented by DIP treatment. There was an increase both iHSP72 and eHSP72 levels in NIHL group, that was avoid by DIP treatment. Also, H-index (plasma/cochlea eHSP72/iHSP72 ratio) was increased in NIHL and prevented by DIP treatment. Our data indicates cochlear damage induced by noise exposition is accompanied by local and systemic heat shock response. Also, alanyl-glutamine supplementation, with the protocol tested herein, did not prevent noise induce hearing loss, but reduces stress markers suggesting further investigations. Finally, plasma levels of 72 kDa heat shock proteins can be used as biomarker of auditory stress condition after noise exposure.

Keywords: hearing loss, heat shock response, iHSP70 eHSP70, noise exposure, alanyl-glutamine, H-index,

LISTA DE ABREVIATURAS

dB NPS: Decibéis nível de pressão sonora
dB: decibéis
DIP: Dipeptídeo alanilglutamina
DNA: ácido desoxirribonucleico
ELISA: Ensaio de imunoabsorção enzimática
EPA: Equipamento de proteção auditiva
ERN: Espécies reativas de nitrogênio
ERO: Espécies reativas de oxigênio
EUA: Estados Unidos da América
FDA: *Food and Drug Administration*
GGA: Geranilgeranilacetona
GSH: Glutationa
HSF1: Fator 1 de choque térmico
HSP: Proteína de choque térmico
HSP70: Proteína de choque térmico de 70 kDa
ISO: Organização internacional para padronização
NPS: Nível de Pressão Sonora
OMS: Organização Mundial de Saúde
PAIR: Perda auditiva induzida pelo ruído
TLR: receptores do tipo *toll-like*
US$: Dólares americanos

Sumário

1 **INTRODUÇÃO**

1.1 **Perda Auditiva**

A perda auditiva é a mais prevalente desordem sensorial e um problema que cresce globalmente. No mundo há aproximadamente 360 milhões de pessoas com perda auditiva, sendo que 90% são adultos (LOOI et al., 2015). Esta desordem leva a um grande impacto no cotidiano das pessoas, principalmente no que tange a comunicação, causando muitas dificuldades durante a vida, diretamente proporcional à sua gravidade. A perda auditiva causa um efeito negativo na habilidade dos indivíduos se comunicarem com outros, e portanto, traz prejuízo ao processo de ensino aprendizagem, na obtenção e manutenção de emprego e nas relações interpessoais, podendo levar a estigmatização e preconceito. O seu impacto não está apenas no indivíduo surdo, mas em toda família e sociedade em que está inserido (LOOI et al., 2015; WORLD HEALTH ORGANIZATION, 2012). A dificuldade de comunicação em decorrência da perda auditiva pode resultar em sensação de solidão, isolamento, frustração e dependência (CIORBA et al., 2012). Com o intuito de demonstrar a importância da audição, Helen Keller, uma autora americana cega e surda, disse: “A cegueira separa as pessoas das coisas, enquanto a surdez separa as pessoas das pessoas”.

Embora muitas das causas de perda auditiva possam ser identificadas e remediadas, milhões de pessoas com perda auditiva ainda vivem em difíceis condições de acesso ao tratamento da perda auditiva, principalmente ao uso de aparelhos auditivos, levando a dificuldade de convívio familiar e social (LOOI et al., 2015). Há muitas causas para a surdez ou perda auditiva, desde fatores genéticos, infecções como a meningite e otite média, exposição a ruído excessivo, envelhecimento, medicações ototóxicas, exposição a substâncias químicas, entre outras (WORLD HEALTH ORGANIZATION, 2012). Muitos destes fatores podem levar a um dano sensorioneural, principalmente ao órgão de Corti localizado na cóclea, onde se localizam as células ciliares externas e internas, fundamentais para o funcionamento da audição. Ao contrário de outros vertebrados, os mamíferos são incapazes de regenerar as células ciliares. Portanto, após um dano que leve a morte celular, há perda auditiva permanente (JIANG; SHA; SCHACHT, 2005; LAYMAN et al., 2015).

1.2 **Perda Auditiva Induzida pelo Ruído**

Dentre as causas de déficit auditivo, a perda auditiva induzida pelo ruído (PAIR) é muito importante no âmbito de saúde ocupacional, principalmente em países industrializados, sendo a segunda causa mais comum de perda auditiva sensorioneural, depois da presbiacusia, caracterizada pela perda auditiva provocada pelo envelhecimento. Além disso, é a maior causa de dano auditivo evitável pelo mundo (SLIWINSKA-KOWALSKA; PAWELCZYK, 2013).

A incidência da PAIR em crianças e adultos jovens está crescendo especialmente pela exposição aos tocadores portáteis de música, com uso de fones de ouvido (DANIEL, 2007). PAIR é a doença ocupacional mais comum nos EUA: cerca de 22 milhões de trabalhadores são expostos a níveis sonoros danosos no trabalho, e anualmente são estimados que US$242 milhões são gastos devido a incapacidade da perda auditiva (BASNER et al., 2014).

PAIR também pode resultar da exposição ao ruído no âmbito recreacional ou militar. O risco de PAIR pode ser diminuído pela redução da exposição do ruído ou pelo uso de equipamento de proteção auditiva individual, chamado equipamento de proteção auditiva (EPA). Contudo, para pessoas em certas ocupações militares e industriais, a exposição ao ruído pode ocorrer em momento não esperado ou o ruído exceder a capacidade de proteção dos protetores auriculares (LO et al., 2013). De acordo com a Comunidade Européia (2004), cerca de 20% dos trabalhadores europeus são expostos a ruídos tão intensos que eles têm que elevar o tom da voz para falar com as outras pessoas, por metade ou mais do seu tempo de trabalho. Se estima que no mundo 500 milhões de indivíduos estejam sob risco de desenvolvimento de PAIR.

A proteção dos trabalhadores contra a exposição ao ruído é objeto de recomendações internacionais, a exemplo da ISO-1999 e de regulamentações específicas de cada país. No Brasil, é obrigatório o uso de protetores auditivos em intensidades sonoras superiores a 85 dB por 8 horas diárias ou dose equivalente. Apesar do uso obrigatório de EPA em trabalhadores expostos ao ruído, a prevalência de uso é baixa, cerca de 42,2% no Brasil e 65,7% nos Estados Unidos (MEIRA; SANTANA; FERRITE, 2015).

Um estudo global demonstrou que os efeitos da exposição ocupacional ao ruído são maiores em homens do que em mulheres em todas as regiões, devido a

diferenças nas categorias ocupacionais, setores de serviço e o tempo de trabalho. Apesar disso, foi visto que se dado o mesmo nível de exposição, todas as pessoas, tanto homens quanto mulheres, desenvolverão perda auditiva semelhante (NELSON et al., 2005).

A participação das mulheres no mercado de trabalho vem aumentando, principalmente em setores de atividade econômica e ocupações tradicionalmente consideradas masculinas, o que pode traduzir maiores níveis de prevalência de PAIR em mulheres. A própria OMS recomenda estudos que investiguem a exposição ao ruído e seus efeitos separadamente entre homens e mulheres, devido a poucos estudos realizados com mulheres (MEIRA; SANTANA; FERRITE, 2015). Um estudo com 299 trabalhadores expostos ao ruído mostrou uma grande diferença no uso de EPA entre os gêneros, sendo que 59,3% dos homens usavam EPA contra 21,4% em mulheres (MEIRA; SANTANA; FERRITE, 2015). Este menor uso de EPA pelas mulheres, mostra sua condição de menor proteção, maior vulnerabilidade e risco potencial de PAIR. Isso se repete mesmo em países desenvolvidos, como nos Estados Unidos, onde o uso de EPA entre as mulheres é de 50,7% e nos homens de 68,9% (TAK; DAVIS; CALVERT, 2009).

Após exposição a um ruído idêntico, a magnitude dos efeitos danosos tem grande variabilidade individual, indicando diferentes níveis de suscetibilidade ao desenvolvimento de PAIR. Essa variabilidade individual tem sido atribuída a complexidade da doença, visto que é resultante da interação de fatores genéticos e comportamentais (SLIWINSKA-KOWALSKA; PAWELCZYK, 2013). Dependendo da suscetibilidade do indivíduo à estimulação acústica, à intensidade e à duração da exposição ao ruído, pode haver uma mudança temporária ou permanente no limiar auditivo (LO et al., 2013).

Sons intensos causam uma dramática mudança no fluxo sanguíneo coclear, incluindo aumento da permeabilidade coclear, vasoconstrição capilar, e estagnação sanguínea em capilares da estria vascular (NUTTALL, 1999). Isto torna as células ciliares relativamente anóxicas. Após restabelecimento do fluxo coclear, o retorno do aporte de oxigênio durante a reperfusão, aumenta o dano secundário metabólico devido às espécies reativas de oxigênio (EROs) (POIRRIER et al., 2010).

Após o trauma acústico, danos mecânicos diretos e alterações metabólicas indiretas ocorrem nas células ciliares da cóclea (WANG; LIBERMAN, 2002). Neste sentido, os mecanismos de proteção antioxidante têm papel fundamental na resposta

bioquímica das células cocleares (HENDERSON et al., 2006). As células ciliares da cóclea respondem ao estresse induzido pelo ruído gerando espécies reativas do oxigênio (ERO) e espécies reativas do nitrogênio (ERN). Níveis excessivamente altos de ERO e ERN podem ultrapassar as defesas antioxidantes celulares, causando estresse oxidativo (desequilíbrio entre a produção de ERO e a capacidade de defesa antioxidante a favor das primeiras) com dano ao DNA, lipídeos e proteínas, com subsequente morte celular e perda auditiva (POIRRIER et al., 2010). Assim, as células de mamíferos respondem ao estresse pela ativação de uma variedade de interações moleculares, sendo que algumas garantem a sobrevivência celular e outras levam à morte celular (FAIRFIELD et al., 2005). O sistema antioxidante coclear inclui a glutationa (GSH), glutationa peroxidase, glutationa redutase, superóxido dismutase e catalase (RYBAK et al., 2009).

A fim de desenvolver novas terapias para prevenção da PAIR, uma prioridade alta é dada para pesquisa objetivando a melhora do conhecimento cientifico das causas moleculares que levam a estas doenças. Em 2015, a *InterAcademy Medical Panel* (IAMP) realizou uma chamada para ação de fortalecimento para o cuidados de saúde voltado para a perda auditiva, citando a importância da realização de pesquisas e programas de inovação nesta patologia (LOOI et al., 2015). O prejuízo e o ônus causado pelas perdas auditivas é muito grande e até hoje não existe droga liberada pela FDA para o tratamento e/ou prevenção da PAIR (BAO et al., 2013).

Frente a isso, muitos estudos vêm sendo realizados com substâncias antioxidantes contra o estresse oxidativo coclear. Em um desses estudos, Lo et al. (2013) demonstraram efeito protetor da D-metionina na perda auditiva permanente induzida pelo ruído. Foram usados cobaios expostos a 105 dB nível de pressão sonora (NPS) durante 6 horas como modelo animal para perda auditiva permanente, gerando uma perda de 10 dB NPS no grupo controle após 14 dias da exposição (LO et al., 2013). Em outro estudo foram utilizados ratos Sprague-Dawley como modelo animal para PAIR. Foi utilizado ruído por banda de oitava centralizado em 4000Hz a 124 dB NPS durante 2 horas. Como resultado, houve uma perda temporária auditiva, 3 dias após o ruído, de 50 dB em relação a antes do ruído. Além disso, foi evidenciada perda auditiva permanente com cerca de 40 dB de diferença do limiar antes do ruído, do sétimo dia até o 3º. mês após ruído. Neste estudo, foi utilizado o potencial evocado auditivo de tronco encefálico com estimulo em forma de cliques para avaliação de limiares auditivos (FUJIOKA et al., 2006).

1.3 **Proteínas de Choque Térmico de 70 kDa – HSP70**

Proteínas de choque térmico (HSP, do inglês *Heat Shock Proteins)* são proteínas altamente conservadas tanto em organismos eucarióticos como em procarióticos. O primeiro relato sobre proteínas expressas em resposta ao calor foi documentado em células de glândulas salivares de *Drosophila buskii* após choque térmico acidental, por Ritossa (JOHNSON; FLESHNER, 2005; RITOSSA, 1962).

HSP são categorizadas em famílias de acordo com seu peso molecular e incluem as subclasses HSP110, HSP100, HSP90, HSP70, HSP60, HSP30 E HSP10 (HENDERSON, 2010). Todas estas famílias de proteínas são consideradas como integrantes da família de proteínas conhecidas como “proteínas de resposta ao estresse”. Estas proteínas tem sua expressão induzida por diferentes tipos de agentes estressores, entre eles, fatores ambientais, patológicos e fisiológicos, assim como exposição a metais pesados, radiação ultravioleta, infecções virais e bacterianas, inflamação, inibidores da ciclooxigenase, estresse oxidativo, fatores de crescimento, choque térmico, isquemia, exercício, estresse metabólico, ruído e drogas (LINDQUIST; CRAIG, 1988; MATHEW; MORIMOTO, 1998; YOSHIDA; KRISTIANSEN; LIBERMAN, 1999). A mais estudada e conservada, devido a sua alta expressão em células de mamíferos sob condições de estresse, é a família de proteínas de choque térmico de 70 kDa (HSP70), que compreendem um número de proteínas com peso molecular de 66 a 78 kDa (HENDERSON, 2010).

Sabe-se que as HSP70 funcionam como chaperonas moleculares intracelulares que facilitam o transporte de proteínas, impedem a agregação de proteínas durante a dobragem e protegem as cadeias polipeptídicas recentemente sintetizadas contra dobragem errônea e desnaturação proteica (HECK; SCHÖLER; DE BITTENCOURT, 2011).

As HSP70 intracelulares (iHSP70) estão descritas como tendo ações moleculares anti-inflamatórias, promovendo citoproteção através de mecanismos antiapoptóticos, inibindo expressão gênica e regulando a progressão do ciclo celular. O efeito das iHSP70 pode ser explicado pela inibição da ativação do fator nuclear κB (NF-κB), o qual tem profundas implicações para imunidade, inflamação, sobrevida

celular e apoptose (BORGES et al., 2012; HECK; SCHÖLER; DE BITTENCOURT, 2011; KRAUSE et al., 2015).

Pensava-se que as HSP tinham funções restritas no ambiente intracelular. Contudo, um número crescente de observações tem indicado que elas podem ser liberadas no espaço extracelular (eHSP70) tendo vários efeitos em outras células (HECK et al., 2017; HECK; SCHÖLER; DE BITTENCOURT, 2011; KRAUSE; RODRIGUES-KRAUSE, 2011).

Um aspecto interessante da fisiologia das HSP70 é sua versatilidade de induzir funções antagônicas, dependendo da sua localização. As iHSP70 tem um potente efeito anti-inflamatório, enquanto as eHSP70 tem papel oposto, induzindo a ativação de diversas vias pró-inflamatórias. A exposição crônica às eHSP70 induz a ativação dessas vias pela ligação aos receptores de membrana como Toll-Like (TLR), embora as eHSP70 também tem sido demonstradas como capazes de atuar como anti-inflamatórias e como fatores imunossupressivos, após internalização e processamento de antígenos (BORGES et al., 2012; HECK; SCHÖLER; DE BITTENCOURT, 2011). Portanto, as HSP70 tem se mostrado como importantes reguladoras do estresse oxidativo, sendo parte da maquinaria antioxidante intracelular, fazendo as iHSP70 ainda mais importantes para inibição da apoptose e inflamação (KRAUSE et al., 2015).

O mecanismo pelo qual diferentes células são capazes de exportar a HSP70 para o ambiente extracelular, ainda é bastante discutido. Ao menos duas fontes de eHSP70 merecem destaque neste contexto: as HSP70 podem ser liberadas como produtos da necrose celular diante de situações de estresse celular irreversível. Em contraste, já foi demonstrado que ocorre a liberação de eHSP70 por mecanismos secretórios diferentes dependendo do tipo celular, em destaque para a presença de vesículas contendo estas proteínas prontas para serem exportadas diante de situações adversas (HECK et al., 2017). Estas vesículas extracelulares podem ativar outros tipos de células, como as células do sistema imune, como parte de mecanismo para evitar ou controlar a propagação de um insulto (DE MAIO, 2014).

Há evidência de correlação entre os níveis sanguíneos de eHSP70 e o prognóstico em pacientes sofrendo de diversas doenças, geralmente relacionadas com estresse oxidativo. Enquanto pessoas saudáveis geralmente tem níveis plasmáticos baixos de eHSP70, a associação do aumento desta proteína com doença ou progressão de doença tem sido hipotetizado. No mesmo sentido, a longevidade e

saúde têm sido atribuídas a baixos níveis de eHSP70. A presença de estresse oxidativo, inflamação, desordens cardiovasculares e fibrose pulmonar foram diretamente correlacionadas com a concentração de eHSP70 no sangue (HECK; SCHÖLER; DE BITTENCOURT, 2011).

Resumidamente podemos considerar que os níveis de eHSP70 estão associados com processos danosos ao organismo, enquanto que a capacidade de responder a situações de estresse celular, aumentando a quantidade de iHSP70, parece ser essencial para a saúde celular. Em decorrência disso, a razão entre eHSP70 e iHSP70 (eHSP72/iHSP70) vem sendo estudada a fim de revelar o estado inflamatório global de um organismo em diferentes situações. Esta razão permite a obtenção de um índice, proposto como índice-H. Assumindo que em situações basais (controle) esta razão seja igual a 1, valores entre 0 a 1 denotam uma resposta anti-inflamatória, enquanto que valores superiores a 1 demonstram um perfil inflamatório, sendo que valores maiores que 5 indicam uma resposta inflamatória intensa (GOETTEMS-FIORIN et al., 2016; HECK, 2011). Este índice nunca foi estudado em relação a PAIR ou em estudos clínicos ou pré-clínicos que investigam novas estratégias terapêuticas para a PAIR.

1.5 **HSP70 na Orelha Interna: Possibilidades Terapêuticas Atuantes nas Vias de Choque Térmico**

A primeira evidência da presença de HSP70 na cóclea foi realizada por Neely et al. (1991). Foi observado que no sistema visual as HSPs são elevadas a níveis que protegem a retina da luz excessiva 18h após estresse térmico (BARBE et al., 1988). No sistema auditivo, o dano causado por ruído interrompido com 18h de período de descanso entre as exposições, reduziu a morte das células ciliares quando comparada ao dano por exposição ao ruído contínuo (BOHNE; ZAHN; BOZZAY, 1985). Assim, os pesquisadores sugeriram a presença de um mecanismo neuroquímico similar na cóclea. Adicionalmente, a descoberta que a estimulação acústica, antes de uma exposição mais intensa, reduziu o dano nas células ciliares na cóclea, também apoiou a presença de mecanismos protetores na cóclea (CANLON; BORG; FLOCK, 1988). Devido a similaridade no curso de tempo seguido do estressor, eles hipotetizaram que, como na retina, mecanismos protetores na cóclea podem ser mediados pelas HSPs. Assim, por imunoistoquímica de cócleas de cobaias, foi evidenciado pela primeira vez

a presença de HSP70 em cócleas normais, não expostas a fatores estressores como o ruído (NEELY; THOMPSON; GOWER, 1991).

Pensando que a capacidade de sobrevivência celular frente ao choque térmico ou outros estressores depende da capacidade de indução de HSP, Dechesne et al. (1992) estudaram se a expressão de HSP72 (forma induzível de 72 kDa da família das HSP70) poderia ser induzida na cóclea por estresse. Utilizaram cobaias e ratos expostos ao choque térmico (elevação da temperatura corporal a 42,5°C por 5 minutos) e evidenciaram indução de HSP72 na cóclea, principalmente na estria vascular (DECHESNE et al., 1992). No mesmo ano, *Myers et al.* (1992) demonstraram elevação de HSP72 na cóclea após isquemia coclear transitória de 10 minutos, com pico máximo 6h após o evento isquêmico (MYERS et al., 1992).

Frente a necessidade de avaliar um fator estressor mais específico e comum da cóclea, *Lim et al.* (1993) estimularam ratos com ruído intenso. Apesar de não realizarem um seguimento por longo prazo da expressão de HSP72 (4, 6 e 8h), foi evidenciada máxima expressão após 6h da cessação do estímulo, que é similar a outros estudos (DECHESNE et al., 1992; THOMPSON; NEELY, 1992). A imunodetecção de HSP72 foi maior nas células ciliares externas (LIM et al., 1993), pois são mais suscetíveis ao trauma acústico que a células ciliares internas (LIBERMAN; BEIL, 1979). Até então, não se sabia o exato papel da HSP70 na cóclea, porém se acreditava que ela tivesse papel importante na proteção celular frente a um agente estressor (LIM et al., 1993).

Foi *Yoshida et al.* (1999) que comprovaram o papel citoprotetor coclear das HSP70. O estudo avaliou que camundongos precondicionados por choque térmico foram protegidos do dano auditivo, 6 e 12h após a terapia térmica em todo o corpo, que foi relacionado com o aumento da expressão coclear de iHSP70 (YOSHIDA; KRISTIANSEN; LIBERMAN, 1999).

O fator 1 de choque térmico (HSF1) é um dos fatores de transcrição responsáveis para regulação de HSP70 induzida pelo estresse. Sob condições normais, HSF1 é mantido em estado monomérico inativo. Na resposta ao estresse, HSF1 monomérico sofre trimerização e um aumento na fosforilação, resultando na sua ativação e na transcrição dos seus genes alvo (MATHEW; MORIMOTO, 1998). Em cócleas de ratos e camundongos sem estresse, HSF1 tem alta expressão em células ciliares, estria vascular e gânglio espiral. *Fairfield et al.* (2002) mostraram presença e ativação de HSF1 em resposta a hipertermia em cócleas de roedores

(FAIRFIELD et al., 2002) e exibe um padrão de expressão que se correlaciona diretamente com o que foi relatado com as HSP70 em outros estudos na cóclea (DECHESNE et al., 1992; LIM et al., 1993; YOSHIDA; KRISTIANSEN; LIBERMAN, 1999). Os resultados mostraram um potencial papel do HSF1 como regulador de resposta ao estresse na cóclea de ratos e camundongos.

O desenvolvimento de um modelo de camundongo deficiente em HSF1 (XIAO et al., 1999) permitiu uma avaliação mais específica do papel das vias de estresse dependentes do HSF1 na cóclea, pois ainda não estava claro se HSF1 era necessário para proteção das células ciliares contra fatores estressores. Para esclarecer isto, *Sugahara et al. (2003)* analizaram camundongos deficientes em HSF1, demonstrando que apesar de possuírem audição normal, tiveram maior perda das células ciliares quando expostos a ruído intenso quando comparados a camundongos nativos, sendo que os HSF1 +/+ perderam 20% de células ciliares externas contra 60% de perda nos HSF1 -/- (SUGAHARA et al., 2003). Assim, se comprovou que a ativação de HSF1 protege as células ciliares do ruído danoso, mostrando a importância das HSP70 na citoproteção coclear.

Na mesma linha, *Fairfield et al.* (2005) expuseram camundongos a nível de ruído que causa perda auditiva temporária somente em HSF1 +/+, sem causar perda permanente (98 dB NPS por 2h). Os camundongos HSF1 -/- tiveram uma perda permanente, 2 semanas após a exposição ao ruído, de cerca de 20 dB, demonstrando a importância das vias de estresse (HSF1 e HSP70) na sobrevida das células ciliares frente a ruído intenso (FAIRFIELD et al., 2005). Também foi avaliado que as HSP70 são necessárias para proteção da morte de células ciliares induzida pela cisplatina em utrículos de camundongos HSF1 -/- (BAKER et al., 2014). Mais tarde, foi avaliado o papel da HSF1 na cóclea após estresse pelo ruído pela análise das HSPs em camundongos HSF1 +/+ e HSF1 -/-, demonstrando rápida indução de HSP25, HSP47, HSP72, HSP73 (forma constitutiva de 73 kDa da família de HSP70), HSP84, HSP86 E HSP110 nas cócleas de camundongos HSF1 +/+, confirmando o papel essencial de HSF1 na mediação da resposta ao choque térmico (GONG et al., 2012).

Ao contrário dos estudos animais, a descoberta de fatores genéticos humanos predispondo a perda auditiva tem encontrado muitas dificuldades. Estudos nas populações chinesa, sueca e polonesa compararam alterações genéticas para síntese de HSP70 entre pessoas sem perda auditiva e com PAIR, que trabalhavam em mesmo ambiente ruidoso. Foram avaliados três genes responsáveis pela síntese da HSP70

(nominadas no trabalho como *HSP70-1*, *HSP70-2* e *HSP70-hom)* (SLIWINSKA-KOWALSKA; PAWELCZYK, 2013). Variações nestes genes foram relacionados com suscetibilidade a PAIR nas 3 populações (KONINGS et al., 2009; YANG et al., 2006).

Com evidências da importância das iHSP70 na proteção coclear, muitos pesquisadores visaram possibilidades terapêuticas para as perdas auditivas sensorioneurais que atuassem nas vias de choque térmico, principalmente na indução coclear de iHSP70. Diversos reagentes como etanol, arsênico e cádmio foram usados para induzir resposta ao choque térmico, ou seja, induzir a síntese de proteínas de estresse como as iHSP70 (LINDQUIST; CRAIG, 1988). Contudo, estas condições de indução de resposta de choque térmico são frequentemente danosas para a audição, tornando clinicamente impossível o uso para proteção auditiva. Portanto, não significa que um agente indutor da síntese coclear de iHSP70 cause um papel citoprotetor, pois a elevação desta proteína pode ser uma resposta ao estresse causado pelo agente nas células sensoriais da cóclea. Uma substância ideal causaria elevação dos níveis de iHSP70 na cóclea sem ter efeito lesivo direto neste órgão.

Pensando nisso, *Mikuriya et al*. (2005) pesquisaram os efeitos da geranilgeranilacetona (GGA) na cóclea. GGA é uma droga oral anti-úlcera péptica que possui efeito protetor devido a sobrerregulação de HSPs na mucosa gástrica, intestino delgado, medula espinal, hepatócitos, coração, cérebro e retina. O propósito do estudo foi investigar se uma dose única de GGA poderia induzir a síntese de HSPs na cóclea e se a administração oral teria efeito protetor para cóclea contra o trauma acústico. Evidenciaram que dose única oral de GGA (600mg/kg) levou a um aumento da expressão de iHSP70 24 a 48h após. Em animais tratados com mesma dose diária, 7 dias antes de uma exposição a ruído (130 dB NPS por 3h), foi observado limiares auditivos significantemente menores que controles, mostrando menor lesão ao órgão de Corti. Além disso, um dano na parede lateral da cóclea produzido pela inoculação de lipopolissacarídeo foi protegido pela pré-tratamento com GGA (SONE et al., 2005).

Na avaliação de outros agressores da orelha interna, *Sano et al.* (2007), usando cultura de cóclea de ratos, avaliaram se a GGA tinha efeito protetor contra a ototoxicidade pela gentamicina. Além de corroborar os achados prévios de indução de iHSP70 pela GGA (MIKURIYA et al., 2005; SONE et al., 2005), foi demonstrado uma maior sobrevida das células ciliares externas com o tratamento com GGA em uma concentração de 10^{-5} M quando expostas a gentamicina (SANO et al., 2007). A GGA também foi benéfica contra a lesão mitocondrial coclear pelo ácido 3-

nitropropiônico em cobaias, com um pré-tratamento com GGA (800 mg/kg por 7 dias), demonstrando melhora significativa do limiar auditivo (KIM et al., 2010).

Avaliando a senescência auditiva, *Mikuriya et al.* (2008) analisaram que na cóclea de camundongos suscetíveis à perda auditiva pela idade (DBA/2J), a expressão de iHSP70 se mostrou reduzida com a idade, enquanto em camundongos controles mostraram aumento desta expressão com 9 meses de idade. Por outro lado, a suplementação alimentar com 0,5% de geranilgeranilacetona (indutor da expressão das HSPs conforme descrito anteriormente), foi capaz de aumentar a expressão coclear de iHSP70 e diminuir a perda auditiva relacionada com a idade (MIKURIYA et al., 2008). Então, alta expressão de iHSP70 na cóclea pode ser importante para manutenção da função auditiva. Dessa forma, a GGA, um comprovado indutor da HSP70, tem a possibilidade de ser segura e útil para tratamento das desordens cocleares, porém ainda não existem estudos em humanos com a GGA visando proteção auditiva.

Drogas terapêuticas com efeitos adversos ototóxicos causam significativa perda auditiva para milhares de pacientes anualmente. Em decorrência disso, há uma necessidade crítica de terapias para proteção da orelha interna sem inibir a eficácia terapêutica dessas drogas. A indução coclear de iHSP70 é uma alternativa para inibição da morte das células ciliares e perda auditiva pelos aminoglicosídeos e cisplatina. O choque térmico em cultura de utrículo de camundongo demonstrou efeito protetor significativo contra a morte das células ciliares por aminoglicosídeo e cisplatina (CUNNINGHAM; BRANDON, 2006). Na análise *in vivo*, utilizando camundongos com expressão aumentada de HSP70, foi demonstrada a importância da expressão de iHSP70 contra a ototoxicidade por aminoglicosídeo (TALEB et al., 2009).

Na procura de alternativas de indução das HSP70 na ototoxicidade, *Roy et al*. (2013) desenvolveram um protocolo de exposição ao ruído sem causar dano permanente na audição. A exposição prévia levou a um aumento da expressão de iHSP70 e protegeu o dano posterior pela ototoxicidade. Assim, a terapia sonora é uma promessa de prevenção da perda auditiva para os pacientes que utilizam aminoglicosídeos e cisplatina, porém ainda faltam ensaios clínicos.

Frente a muitas evidências do papel citoprotetor das HSP70, a sua indução farmacológica pode ser uma abordagem viável para proteção das células sob estresse. Recentemente, um consórcio de 26 laboratórios cooperados com a agência

norteamericana *Food and Drug Administration* (FDA) aprovou drogas e compostos bioativos a fim de identificar compostos que ativem a resposta do choque térmico (FRANCIS et al., 2011).

No intuito de induzir o aumento da expressão de iHSP70, consistentes achados demonstram a habilidade das eHSP70 serem internalizadas pelas células (BORGES et al., 2012). As ações das eHSP70 tem sido demonstradas serem mediadas pela via do NF-kB após ligação com os TLRs (ASEA, 2002), porém estes são recepetores de sinalização, e não de endocitose. Receptores *scavenger* e lecitina-*like* foram vistos como receptores de endocitose internalizando as eHSP70, porém os eventos que seguem após a ligação com estes receptores, ainda não estão bem caracterizados (BORGES et al., 2012).

Outro estudo demonstrou que a ativação da via do NF-κB, através de um inibidor da histona desacetilase, foi responsável por um aumento da expressão das iHSP70 e protegeu contra um dano auditivo causado por aminoglicosídeos em camundongos (LAYMAN et al., 2015).

Frente a isso, as evidências citadas demonstram que a indução das iHSP70 pelas próprias eHSP70 pode ser possível por algumas vias, desde sua internalização através de receptores de endocitose à ativação de sinalização de membrana celular através dos TLRs com ativação intracelular do NF-κB. Esta interação das eHSP70 com o TLR, parece ser realizada com os TLR 2 e 4 nas células apresentadoras de antígenos, exercendo uma função imunoreguladora, incluindo a superexpressão de moléculas de adesão e liberação de citocinas e quimiocinas (DE MAIO, 2014).

Além disso, as HSP são superexpressas em diversas doenças neurodegenerativas e podem eficientemente corrigir a condição inflamatória no sistema nervoso central causado pelo estresse oxidativo e proteínas mal formadas (YURINSKAYA et al., 2015).

Um grupo de pesquisa russo demonstrou que a administração de eHSP70 purificadas de origem humana, intranasal, teve efeitos benéficos quando utilizada em dois modelos independentes de neurodegeneração da doença de Alzheimer, em camundongos (BOBKOVA et al., 2014). Também demonstrou que as eHSP70 administradas via intracerebroventricular, tiveram grande atividade neuroprotetora, diminuindo crises epiléticas em modelo animal de ratos (EKIMOVA et al., 2010).

A descoberta das eHSP70 e a possibilidade de expressar e isolar grandes quantidades de HSP70 recombinante intactas, associado com resultados

experimentais promissores, fazem desta proteína uma atrativa opção para o desenvolvimento de drogas anti-inflamatórias eficientes e drogas que podem tratar várias doenças neurodegenerativas (YURINSKAYA et al., 2015).

Embora estes estudos anteriormente citados demonstrem que a administração de eHSP70 recombinantes tem benefícios em neuroproteção, não está claro se as eHSP70 se preservam intactas após entrar nas células e quais fatores são responsáveis pela sua internalização e degradação dentro das células (YURINSKAYA et al., 2015).

Outra opção promissora é o uso de substâncias com efeito potencializador da expressão de HSP70, como é o caso da L-glutamina. Ela está associada com potencialização da expressão de iHSP70, tanto *in vitro* (HAMIEL et al., 2009) como *in vivo* (SINGLETON; WISCHMEYER, 2007). Através do HSF1, L-glutamina é capaz de facilitar a transcrição e síntese de iHSP70 em um processo que depende, pelo menos parcialmente, da ativação da via da glucosamina (HAMIEL et al., 2009). Contudo, as vias pelas quais a L-glutamina aumenta a expressão de HSP70 são na maioria desconhecidas.

Do ponto de vista nutricional, a L-glutamina é o aminoácido livre mais abundante no corpo sendo primariamente produzido pelos músculos esqueléticos e lançado no sangue (NEWSHOLME et al., 2003). Frente a um estresse oxidativo celular, para proteção contra as espécies reativas, o tripeptídeo GSH é o mais importante antioxidante intracelular solúvel não-enzimático e tem muitas funções metabólicas e de proteção no metabolismo celular incluindo atenuação do estresse oxidativo (ROTH, 2008). Evidências experimentais sugerem que metade da L-glutamina necessária para a nova síntese do GSH, é principalmente derivada de uma variedade de tecidos (NEWSHOLME et al., 2003). Contudo, a disponibilidade de L-glutamina intracelular é influenciada pela acessibilidade para o transporte de L-glutamina para dentro da célula. Então, uma redução da L-glutamina, observada em situações de estresse intenso, podem reduzir a concentração de GSH, deixando o corpo mais vulnerável ao estresse oxidativo e morte celular (KIM; WISCHMEYER, 2013).

Com intuito de melhorar o aporte de L-glutamina sobre estresse fisiológico, muitos pesquisadores têm estudado a suplementação dietética deste aminoácido (KIM; WISCHMEYER, 2013). Portanto, o uso oral tanto do dipeptídeo L-glutamina como alanilglutamina (ROGERO et al., 2006) ou soluções contendo L-glutamina e L-

alanina, ambos em suas formas livres, vem sendo uma alternativa efetiva não invasiva para aumentar os níveis de L-glutamina no corpo (CRUZAT et al., 2014). Petry et al. (2014) demonstraram que a suplementação de L-glutamina influencia no estoque intracelular de GSH e aumento das iHSP70 em tecido muscular de ratos, diminuindo a vulnerabilidade tecidual ao estresse oxidativo (PETRY et al., 2014).

Ogushi et al. (2012) demonstraram a presença de um transportador de glutamina SAT1 nas células ciliares internas, além da presença de glutaminase, mostrando a via de captação de glutamina nas células ciliares para formação do glutamato (OGUCHI et al., 2012). Além disso, com este fato pode ser hipotetizado que a glutamina pode ser transportada para células do órgão de Corti, com a possibilidade de síntese de GSH através da glutamina, diminuindo o estresse oxidativo coclear e um dano auditivo, seja ela causado por um ruído ou um agente ototóxico, como a cisplatina.

2. **OBJETIVOS**

2.1 Objetivo Geral

- Verificar se a administração do dipeptídeo alanilglutamina é capaz de induzir o aumento da expressão de iHSP72 na cóclea, e deste modo, gerar citoproteção coclear diante do estresse causado pelo ruído, evitando a ocorrência de dano auditivo.

2.2 Objetivos específicos

- Revisar o papel das HSP70 nas perdas auditivas sensorioneurais
- Avaliar se a exposição ao ruído altera a concentração de HSP72 intracoclear (iHSP72) e plasmática (eHSP72);
- Avaliar se eHSP72 pode ser potencial biomarcador de dano auditivo pelo ruído
- Avaliar se o tratamento com alanilglutamina pode alterar a concentração de HSP72 intracoclear (iHSP72) e plasmática (eHSP72) prevenir a perda auditiva induzida pelo ruído;
- Avaliar o índice-H em situação de exposição ao ruído e/ou tratamento com alanilglutamina.

3. **ARTIGOS**

3.1. Artigo 1. Hearing Research (short review). The role of 70 kDa heat shock proteins (HSP70) in sensorineural hearing loss

The role of 70 kDa heat shock proteins (HSP70) in sensorineural hearing loss

Marcos Soares[1,*] and Thiago Gomes Heck[1]

[1]Research Group in Physiology, Post Graduate Program in Integral Attention to Health (PPGAIS-UNIJUI/UNICRUZ), Department of Life Sciences, Regional University of Northwestern Rio Grande do Sul State (UNIJUI), Ijuí, RS, Brazil.
*Corresponding author: rua do comércio, 3000 – dcvida/unijuí, Ijuí, RS, CEP 98700-000 Brazil. Tel.: +55 (55) 33320476. e-mail: marcossoaresorl@gmail.com (M. Soares)

Abstract

Hearing loss is a prevalent sensory disorder in humans impacting on individual ability to oral communication. Many factors can lead to sensorineural damage, mainly to the Corti organ. Faced with stress situations on inner ear, HSP70, an important cytoprotective protein, is activated. Intracellular HSP70s (iHSP70) are described as having anti-inflammatory molecular actions, promoting cytoprotection through anti-apoptotic mechanisms, inhibiting gene expression and regulating cell cycle progression. Therefore, evidences substances or conditions that are able to induce iHSP70 expression in cochlea is a promising field for development of strategies for prevention and treatment of diseases of auditory system.

Key words: Hearing loss, Heat shock protein, HSP70.

1. Introduction

Hearing loss is the most prevalent sensory disorder and a growing problem globally (Looi et al., 2015). Over 5% of the world's population - 360 million people - have disabling hearing loss, with 328 million adults and 32 million children (WHO, 2017). Hearing problems can cause many difficulties in life, the extent of which are directly linked to its severity. One of the main impacts is on the individual's ability to communicate with others. Development of

spoken language is often delayed in children with hearing loss, leading to worsening school performance and psychological disorders. In adults and elderly patients, social isolation is common, causing feelings of loneliness and frustration. This negatively affects mental health, participation in interpersonal relationships, quality of life, and work and career possibilities (Davis et al., 2016; WHO, 2017; Wroblewska-Seniuk et al., 2016). Hearing loss of an individual also impacts the whole family and the wider community (Looi et al., 2015). The World Health Organization estimates that hearing loss has a global annual cost of $750 billion, not including the cost of hearing aids (WHO, 2017).

Hearing loss may be a result of genetic causes, complications at birth, infectious diseases, chronic ear infections, use of ototoxic drugs, exposure to excessive noise, and aging (WHO, 2017). Many of these factors can lead to sensorineural damage, mainly of the Corti organ of cochlea where the outer and inner hair cells, fundamental to functioning of hearing, are found. In mammals, unlike other vertebrates, permanent hearing loss occurs due to an inability to regenerate damaged hair cells (Jiang et al., 2005; Layman et al., 2015).

Upon exposure of the inner ear to stress situations, an important cytoprotective feature of cochlea is activated which involves the expression of 70 kDa heat shock proteins (HSP70 or HSPA). Studies in mice preconditioned by heat-shock have shown that cochlear HSP70 expression increases, and this protects the animals from subsequent noise-induced damage (Yoshida et al., 1999). Heat shock transcription factor 1 (HSF1), regulates the expression of HSP72 (inducible form of HSP70). Fairfield et al. (2005) demonstrated that the suppression of HSF1 expression in HSF1-knockout mice, caused significant noise-induced hearing loss. This highlights the importance of HSP72 expression in cochlear cytoprotection against oxidative stress (Fairfield et al., 2005). In addition, an increase in intracellular expression of HSP70 (iHSP70) is thought to represent cytoprotection by anti-inflammatory mechanisms (Heck et al., 2011).

Based on cytoprotection in the cochlea, many studies have been performed with the aim of inducing the expression of HSPs (Baker et al., 2014; Cunningham and Brandon, 2006; Lim et al., 1993; Liu et al., 2015; Taleb et al., 2009). Knowledge of the molecular role of HSP70 in the inner ear is fundamental to the development of new therapies. Oral drugs are expected to become viable in the next 10 years, but to-date, no drug has been released by the Food and Drug Administration (FDA) for the

treatment and/or prevention of ototoxicity and noise-induced hearing loss (Bao et al., 2013; Mukherjea et al., 2014).

2. Heat shock protein 70 kDa (HSP70)

HSPs are highly conserved proteins in both eukaryotic and prokaryotic organisms. They were first documented in *Drosophila buskii* salivary gland cells after accidental heat shock in 1962 (Johnson and Fleshner, 2005; Ritossa, 1962).

HSPs are categorized into families according to their molecular weight, and include subclasses HSP110, HSP100, HSP90, HSP70, HSP60, HSP30 and HSP10 (Henderson, 2010). They are considered part of a family of proteins known as "stress proteins" because many types of stressors induce their expression. These stressors include environmental, pathological and physiological factors, as well as exposure to heavy metals, ultraviolet radiation, viral and bacterial infections, inflammation, cyclooxygenase inhibitors, oxidative stress, growth factors, heat shock, ischemia, exercise, metabolic stress, noise and drugs (Lindquist and Craig, 1988). The most studied HSP group, due to its high expression in mammalian cells under stress conditions, is the 70 kDa family (HSP70), which comprise a number of proteins with molecular weights of 66 to 78 kDa (Henderson, 2010).

HSP70s are known to function as intracellular molecular chaperones that facilitate protein transport, prevent protein aggregation during folding, and protect newly synthesized polypeptide chains against misfolding and protein denaturation (Heck et al., 2011).

Intracellular HSP70s (iHSP70) exhibit anti-inflammatory molecular actions, by promoting cytoprotection through anti-apoptotic mechanisms, inhibiting gene expression and regulating cell cycle progression. The effect of iHSP70 is due to the inhibition of nuclear factor kB (NF-kB) activation, which has profound implications for immunity, inflammation, cell survival and apoptosis (Borges et al., 2012; Krause et al., 2015).

HSPs were thought to have restricted functions in the intracellular environment. However, a growing number of studies have indicated that they are released into extracellular spaces (eHSP70), where they affect other cells in various ways (Heck et al., 2017, 2011; Krause and Rodrigues-Krause, 2011).

An interesting and versatile aspect of the physiology of HSP70 is its ability to induce antagonistic functions, depending on its location. iHSP70 has a potent anti-inflammatory effect, whilst eHSP70 has the opposing role of inducing the activation of several pro-inflammatory pathways. Chronic exposure to eHSP70 induces the activation of these pathways by binding to membrane receptors such as Toll-Like (TLR). However, eHSP70 is also capable of acting as an anti-inflammatory and immunosuppressive factor after internalization and antigen processing (Borges et al., 2012; Heck et al., 2011). Therefore, HSP70 is an important regulator of oxidative stress, as part of the intracellular antioxidant machinery, and renders iHSP70 even more important for the inhibition of apoptosis and inflammation (Krause et al., 2015).

3. HSP70 in inner ear

Neely et al. (1991) provided the first evidence of the presence of HSP70 in cochlea. In a visual system, HSPs were shown to be elevated to levels that protect retina from excessive light, 18 h after heat-stress (Barbe et al., 1988). In the auditory system, hair cell death due to interrupted noise, with 18 h resting periods between exposures, was lower than cell death due to continuous noise (Bohne et al., 1985). Thus, researchers have suggested the presence of a similar neurochemical mechanism in the cochlea. Additionally, the discovery that acoustic stimulation, before more intense exposure, led to reductions in damage to cochlea hair cells, also indicates a protective mechanism in this organ (Canlon et al., 1988). Due to these finds, the authors hypothesized that protective mechanisms in cochlea are mediated by HSPs. In this way, using immunohistochemistry, the presence of HSP70 in cochlea of guinea pigs not exposed to stressors such as noise, was demonstrated for the first time (Neely et al., 1991).

Considering that cell-survival from heat-shock or other stressors depends on the induction capacity of HSPs, Dechesne et al. (1992) explored whether HSP72 expression in cochlea is induced by stress. They used guinea pigs and rats exposed to heat-shock (elevation of body temperature to 42.5°C for 5 minutes), and showed induction of cochlear HSP72, mainly in stria vascularis. In the same year, Myers et al. (1992), demonstrated an elevation of HSP72 levels in cochlea after transient cochlear ischemia for 10 minutes, with a peak observed 6 h after the ischemic event.

Lim et al. (1993) wanted to evaluate a more specific and common stressor factor of the cochlea, so stimulated rats with intense noise. Although HSP72 expression was not measured long-term (but only after 4, 6 and 8 h), maximal expression was observed 6 h after cessation of stimulus, which is in agreement with other studies (Dechesne et al., 1992; Thompson and Neely, 1992). Using immunodetection, HSP72 levels were found to be higher in outer hair cells (Lim et al., 1993), as they are more susceptible to acoustic trauma than the inner cells (Liberman and Beil, 1979). The exact role of HSP70 in the cochlea remains unclear, but it is believed to have an important role in cell protection against stressors (Lim et al., 1993).

Yoshida et al. (1999) demonstrated the cytoprotective role of HSP70 in the cochlea. They found that mice preconditioned by heat-shock were protected (throughout the body) from auditory damage after 6 and 12 hours, which was attributed to increased cochlear iHSP70 expression.

Heat shock factor 1 (HSF1) is one of the transcription factors responsible for stress-induced regulation of HSP70. Under normal conditions, HSF1 is maintained in an inactive monomeric state. In response to stress, monomeric HSF1 undergoes trimerization and an increase in phosphorylation, resulting in activation of HSF1 and transcription of its target genes (Mathew and Morimoto, 1998). In cochlea of rats and mice without stress, HSF1 has high expression in hair cells, stria vascularis and spiral ganglion. Fairfield et al. (2002) demonstrated

the presence and activation of HSF1 in response to hyperthermia in rodent cochlea. The resulting expression pattern was found to correlate directly with the findings of several other studies (Dechesne et al. 1992; Lim et al., 1993; Yoshida et al., 1999). These results highlight a potential role of HSF1 as a regulator of stress response in the cochlea.

The development of a mouse model deficient in HSF1 (Xiao et al., 1999) allowed the specific evaluation of the role of HSF1-dependent stress pathways in cochlea. At that time, it was still unclear whether HSF1 was required for protection of hair cells against stressors. To clarify this, Sugahara et al. (2003) analyzed HSF1-deficient mice with normal hearing, and demonstrated that a greater loss of hair cells occurred when exposed to intense noise (130 dB for 3 h), when compared to native mice (HSF1 +/+, 20% loss; HSF1 -/- 60% loss). Thus, it is evident that activation of HSF1 protects hair cells from harmful noise, illustrating the importance of HSP70 in cochlear cytoprotection. Similarly, Fairfield et al. (2005) exposed mice to a noise level (98 dB SPL for 2 h) that caused temporary hearing loss only in HSF1 +/+. HSF1 -/- mice had permanent loss of hearing from 20 dB, 2 weeks post-exposure. This confirms the importance of stress pathways (HSF1 and HSP70) in hair cell survival from severe noise. It has also been shown that HSP70 protects cisplatin-induced hair cell death in HSF1 -/- mice (Baker et al., 2014). The role of HSF1 in cochlea after noise stress has been evaluated by HSP analysis in HSF1 +/+ and HSF1 -/- mice (Gong et al., 2012). The authors observed rapid induction of HSP25, HSP47, HSP72, HSP73 (constitutive form of 73 kDa family of HSP70), HSP84, HSP86 and HSP110 in HSF1 +/+ mouse cochlea, confirming the essential role of HSF1 in mediating the heat-shock response.

The identification of human genetic factors predisposing to hearing loss has encountered many difficulties. Studies in Chinese, Swedish, and Polish populations have compared genetic alterations in HSP70 synthesis in people without hearing loss, with Noise-Induced Hearing Loss (NIHL) who worked in the same noisy environment (Sliwinska-Kowalska and Pawelczyk, 2013; Konings et al., 2009; Yang et al., 2006). Three genes responsible for the synthesis of HSP70 (HSP70-1, HSP70-2 and HSP70-hom) were evaluated and variations in these genes were associated with susceptibility to NIHL in these three populations.

Provided with evidence of the importance of iHSP70 in cochlear protection, many researchers have explored the therapeutic potential of heat-shock pathways in sensorineural hearing loss, especially in the cochlear induction of iHSP70. Several reagents such as ethanol, arsenic and cadmium have been used to induce a heat-shock response (Lindquist and Craig, 1988). However, these induction methods are often harmful to hearing; they may induce the cochlear synthesis of iHSP70 due to stress of the sensory cells of cochlea, caused by the agent itself. An ideal therapeutic substance would promote elevation of iHSP70 levels in cochlea, without directly damaging the organ.

Considering these problems, Mikuriya et al. (2005) investigated the effects of geranylgeranylacetone (GGA) on cochlea. GGA is an oral peptic anti-ulcer drug that exhibits a protective effect due to over-regulation of HSPs in the gastric mucosa, small intestine, spinal cord, hepatocytes, heart, brain and retina. The purpose of the study was to assess whether a single dose of GGA induces the synthesis of HSPs in cochlea, and if oral administration has a protective effect on cochlea against acoustic trauma. They reported that a single oral dose of GGA (600 mg/kg) led to an increase in the expression of iHSP70 after 24 to 48 h. In animals treated with the same daily dose for 7 days before a noise exposure (130 dB SPL for 3 h), auditory thresholds significantly lower than the controls were observed, with reduced injury to the Corti organ. In addition, damage to the lateral wall of cochlea produced by lipopolysaccharide inoculation was alleviated by pre-treatment with GGA (Sone et al., 2005).

Sano et al. (2007), used a rat cochlea culture to evaluated whether GGA had a protective effect against gentamicin ototoxicity. Their findings corroborated previous studies into the induction of iHSP70 by GGA (Mikuriya et al., 2005; Sone et al., 2005), and revealed a higher survival of gentamicin-exposed outer hair cells using GGA at a concentration of 10^{-5} M. GGA (800 mg/kg for 7 days) was also shown to be beneficial for cochlear mitochondrial 3-nitropropionic injuries in guinea pigs, with significant improvements in auditory threshold (Kim et al., 2010).

In an evaluation of auditory senescence, Mikuriya et al. (2008) analyzed the cochlea of mice susceptible to age-related hearing loss (DBA / 2J), and showed that expression of iHSP70 reduced with age; the control mice showed an increase in expression at the age of 9 months. On the other hand, food supplementation with 0.5% GGA led to an increase in cochlear expression of iHSP70, and a decrease in age-related hearing loss. Thus, high expression of iHSP70 in cochlea may be important for maintaining auditory function. GGA, a proven inducer of HSP70, has the potential to be a safe and effective treatment for cochlear disorders, although no studies of this nature have been undertaken in humans at the time of writing.

Therapeutic drugs with adverse ototoxic effects cause significant globally hearing loss for thousands of patients annually. As a result, there is a critical need of therapies for inner ear protection, without inhibiting the therapeutic efficacy of these drugs. Cochlear induction of iHSP70 may provide an alternative treatment through the inhibition of hair cell death and hearing loss caused by aminoglycosides and cisplatin. Heat-shock in a mouse utricle culture revealed a significant protective effect against death of hair cells caused by aminoglycoside and cisplatin (Cunningham and Brandon, 2006). *In vivo* analysis, using mice with increased expression of HSP70, also demonstrated the importance of iHSP70 expression in protection from aminoglycoside ototoxicity (Taleb et al., 2009). Alternative techniques of inducing HSP70 in the presence of ototoxicity would be beneficial. Roy et al. (2013) developed such a protocol for exposure to noise without causing permanent hearing damage. Previous noise exposure

was used to induce increased iHSP70 expression, which provided protection against damage due to ototoxicity. Sound therapy therefore shows promise for hearing loss prevention in patients on aminoglycosides and cisplatin, although clinical trials are still lacking.

4. Conclusions and perspectives

The cytoprotective role of cochlear iHSP70 has been established. The use of substances or conditions that induce the expression of these proteins in cochlea is a promising method of preventing and/or treating the pathologies that cause damage to the inner ear. Studies that investigate the role of eHSP70 in cochlear physiology may facilitate the development of new therapeutic approaches, or shed light on the pathophysiological mechanisms surrounding hearing loss.

5. References

Baker, T.G., Roy, S., Brandon, C.S., Kramarenko, I.K., Francis, S.P., Taleb, M., Marshall, K.M., Schwendener, R., Lee, F.-S., Cunningham, L.L., 2014. Heat Shock Protein-Mediated Protection Against Cisplatin-Induced Hair Cell Death. J. Assoc. Res. Otolaryngol. 16, 67–80. doi:10.1007/s10162-014-0491-7

Bao, J., Hungerford, M., Luxmore, R., Ding, D., Qiu, Z., Lei, D., Yang, A., Liang, R., Ohlemiller, K.K., 2013. Prophylactic and therapeutic functions of drug combinations against noise-induced hearing loss. Hear. Res. 304, 33–40. doi:10.1016/j.heares.2013.06.004

Barbe, M.F., Tytell, M., Gower, D.J., Welch, W.J., 1988. Hyperthermia protects against light damage in the rat retina. Science 241, 1817–20.

Bohne, B.A., Zahn, S.J., Bozzay, D.G., 1985. Damage to the Cochlea following Interrupted Exposure to Low Frequency Noise. Ann. Otol. Rhinol. Laryngol. 94, 122–128. doi:10.1177/000348948509400205

Borges, T.J., Wieten, L., Van Herwijnen, M.J.C., Broere, F., Van derZee, R., Bonorino, C., Van Eden, W., 2012. The anti-inflammatory mechanisms of Hsp70. Front. Immunol. 3, 1–12. doi:10.3389/fimmu.2012.00095

Canlon, B., Borg, E., Flock, A., 1988. Protection against noise trauma by pre-exposure to a low level acoustic stimulus. Hear. Res. 34, 197–200.

Cunningham, L.L., Brandon, C.S., 2006. Heat shock inhibits both aminoglycoside- and cisplatin-induced sensory hair cell death. JARO - J. Assoc. Res. Otolaryngol. 7, 299–307. doi:10.1007/s10162-006-0043-x

Davis, A., McMahon, C.M., Pichora-Fuller, K.M., Russ, S., Lin, F., Olusanya, B.O.,

Chadha, S., Tremblay, K.L., 2016. Aging and hearing health: The life-course approach. Gerontologist 56, S256–S267. doi:10.1093/geront/gnw033

Dechesne, C.J., Kim, H.N., Nowak, T.S., Wenthold, R.J., 1992. Expression of heat shock protein, HSP72, in the guinea pig and rat cochlea after hyperthermia: Immunochemical and in situ hybridization analysis. Hear. Res. 59, 195–204. doi:10.1016/0378-5955(92)90116-5

Fairfield, D.A., Kanicki, A.C., Lomax, M.I., Altschuler, R.A., 2002. Expression and localization of heat shock factor (Hsf) 1 in the rodent cochlea. Hear. Res. 173, 109–118. doi:10.1016/S0378-5955(02)00607-X

Fairfield, D. a., Lomax, M.I., Dootz, G. a., Chen, S., Galecki, A.T., Benjamin, I.J., Dolan, D.F., Altschuler, R. a., 2005. Heat shock factor 1-deficient mice exhibit decreased recovery of hearing following noise overstimulation. J. Neurosci. Res. 81, 589–596. doi:10.1002/jnr.20417

Gong, T.W., Fairfield, D. a., Fullarton, L., Dolan, D.F., Altschuler, R. a., Kohrman, D.C., Lomax, M.I., 2012. Induction of heat shock proteins by hyperthermia and noise overstimulation in Hsf1?/? mice. JARO - J. Assoc. Res. Otolaryngol. 13, 29–37. doi:10.1007/s10162-011-0289-9

Heck, T.G., Schöler, C.M., de Bittencourt, P.I.H., 2011. HSP70 expression: Does it a novel fatigue signalling factor from immune system to the brain? Cell Biochem. Funct. 29, 215–226. doi:10.1002/cbf.1739

Heck, T.G., Scomazzon, S.P., Nunes, P.R., Schöler, C.M., da Silva, G.S., Bittencourt, A., Faccioni-Heuser, M.C., Krause, M., Bazotte, R.B., Curi, R., Homem de Bittencourt, P.I., 2017. Acute exercise boosts cell proliferation and the heat shock response in lymphocytes: correlation with cytokine production and extracellular-to-intracellular HSP70 ratio. Cell Stress Chaperones 1–21. doi:10.1007/s12192-017-0771-3

Henderson, B., 2010. Integrating the cell stress response: a new view of molecular chaperones as immunological and physiological homeostatic regulators. Cell Biochem. Funct. 28, 1–14. doi:10.1002/cbf.1609

Jiang, H., Sha, S.-H., Schacht, J., 2005. NF-?B pathway protects cochlear hair cells from aminoglycoside-induced ototoxicity. J. Neurosci. Res. 79, 644–651. doi:10.1002/jnr.20392

Johnson, J.D., Fleshner, M., 2005. Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72. J. Leukoc.

Biol. 79, 425–434. doi:10.1189/jlb.0905523

Kim, Y.H., Song, J.J., Kim, Y.C., Park, K.T., Lee, J.H., Choi, J.M., Lee, J.H., Oh, S.H., Chang, S.O., 2010. Geranylgeranylacetone ameliorates acute cochlear damage caused by 3-nitropropionic acid. Neurotoxicology 31, 317–325. doi:10.1016/j.neuro.2010.03.001

Konings, A., Van Laer, L., Michel, S., Pawelczyk, M., Carlsson, P.-I., Bondeson, M.-L., Rajkowska, E., Dudarewicz, A., Vandevelde, A., Fransen, E., Huyghe, J., Borg, E., Sliwinska-Kowalska, M., Van Camp, G., 2009. Variations in HSP70 genes associated with noise-induced hearing loss in two independent populations. Eur. J. Hum. Genet. 17, 329–335. doi:10.1038/ejhg.2008.172

Krause, M., Heck, T.G., Bittencourt, A., Scomazzon, S.P., Newsholme, P., Curi, R., Ivo, P., Bittencourt, H. De, 2015. The Chaperone Balance Hypothesis : The Importance of the Extracellular to Intracellular HSP70 Ratio to Inflammation-Driven Type 2 Diabetes , the Effect of Exercise , and the Implications for Clinical Management 2015.

Krause, M., Rodrigues-Krause, J.D.C., 2011. Extracellular heat shock proteins (eHSP70) in exercise: Possible targets outside the immune system and their role for neurodegenerative disorders treatment. Med. Hypotheses 76, 286–290. doi:10.1016/j.mehy.2010.10.025

Layman, W.S., Williams, D.M., Dearman, J.A., Sauceda, M.A., Zuo, J., 2015. Histone deacetylase inhibition protects hearing against acute ototoxicity by activating the Nf-κB pathway. Cell death Discov. 1. doi:10.1038/cddiscovery.2015.12

Liberman, M.C., Beil, D.G., 1979. Hair cell condition and auditory nerve response in normal and noise-damaged cochleas. Acta Otolaryngol. 88, 161–76.

Lim, H.H., Jenkins, O.H., Myers, M.W., Miller, J.M., Altschuler, R. a, 1993. Detection of HSP 72 synthesis after acoustic overstimulation in rat cochlea. Hear. Res. 69, 146–150. doi:10.1016/0378-5955(93)90102-7

Lindquist, S., Craig, E.A., 1988. The Heat-Shock Proteins. Annu. Rev. Genet. 22, 631–677. doi:10.1146/annurev.ge.22.120188.003215

Liu, Y., Yu, Y., Chu, H., Bing, D., Wang, S., Zhou, L., Chen, J., Chen, Q., Pan, C., Sun, Y., Cui, Y., 2015. 17-DMAG induces Hsp70 and protects the auditory hair cells from kanamycin ototoxicity in vitro. Neurosci. Lett. 588, 72–7. doi:10.1016/j.neulet.2014.12.060

Looi, L.M., Ganten, D., McGrath, P.F., Gross, M., Griffin, G.E., 2015. Hearing loss: a

global health issue. Lancet 385, 943–944. doi:10.1016/S0140-6736(15)60208-2

Mathew, A., Morimoto, R.I., 1998. Role of the heat-shock response in the life and death of proteins. Ann. N. Y. Acad. Sci. 851, 99–111. doi:10.1111/j.1749-6632.1998.tb08982.x

Mikuriya, T., Sugahara, K., Sugimoto, K., Fujimoto, M., Takemoto, T., Hashimoto, M., Hirose, Y., Shimogori, H., Hayashida, N., Inouye, S., Nakai, A., Yamashita, H., 2008. Attenuation of progressive hearing loss in a model of age-related hearing loss by a heat shock protein inducer, geranylgeranylacetone. Brain Res. 1212, 9–17. doi:10.1016/j.brainres.2008.03.031

Mikuriya, T., Sugahara, K., Takemoto, T., Tanaka, K., Takeno, K., Shimogori, H., Nakai, A., Yamashita, H., 2005. Geranylgeranylacetone, a heat shock protein inducer, prevents acoustic injury in the guinea pig. Brain Res. 1065, 107–114. doi:10.1016/j.brainres.2005.10.045

Mukherjea, D., Ghosh, S., Bhatta, P., Sheth, S., Tupal, S., Borse, V., Brozoski, T., Sheehan, K.E., Rybak, L.P., Ramkumar, V., 2014. Early investigational drugs for hearing loss. Expert Opin. Investig. Drugs 24, 1–17. doi:10.1517/13543784.2015.960076

Myers, M.W., Quirk, W.S., Rizk, S.S., Miller, J.M., Altschuler, R.A., 1992. Expression of the Major Mammalian Stress Protein in the Rat Cochlea Following Transient Ischemia. Laryngoscope 102, 981–987. doi:10.1288/00005537-199209000-00005

Neely, J.G., Thompson, A.M., Gower, D.J., 1991. Detection and localization of heat shock protein 70 in the normal guinea pig cochlea. Hear. Res. 52, 403–406.

Radons, J., 2016. The human HSP70 family of chaperones: where do we stand? Cell Stress Chaperones. doi:10.1007/s12192-016-0676-6

Ritossa, F., 1962. A new puffing pattern induced by temperature shock and DNP in drosophila. Experientia 18, 571–573. doi:10.1007/BF02172188

Roy, S., Ryals, M.M., Van Den Bruele, A.B., Fitzgerald, T.S., Cunningham, L.L., 2013. Sound preconditioning therapy inhibits ototoxic hearing loss in mice. J. Clin. Invest. 123, 4945–4949. doi:10.1172/JCI71353

Sano, H., Yoneda, S., Iwase, H., Itoh, A., Hashimoto, D., Okamoto, M., 2007. Effect of geranylgeranylacetone on gentamycin ototoxicity in rat cochlea culture. Auris Nasus Larynx 34, 1–4. doi:10.1016/j.anl.2006.05.020

Sliwinska-Kowalska, M., Pawelczyk, M., 2013. Contribution of genetic factors to noise-

induced hearing loss: a human studies review. Mutat. Res. 752, 61–5. doi:10.1016/j.mrrev.2012.11.001

Sone, M., Hayashi, H., Yamamoto, H., Hoshino, T., Mizushima, T., Nakashima, T., 2005. Upregulation of HSP by geranylgeranylacetone protects the cochlear lateral wall from endotoxin-induced inflammation. Hear. Res. 204, 140–146. doi:10.1016/j.heares.2005.01.012

Sugahara, K., Inouye, S., Izu, H., Katoh, Y., Katsuki, K., Takemoto, T., Shimogori, H., Yamashita, H., Nakai, A., 2003. Heat shock transcription factor HSF1 is required for survival of sensory hair cells against acoustic overexposure. Hear. Res. 182, 88–96. doi:10.1016/S0378-5955(03)00180-1

Taleb, M., Brandon, C.S., Lee, F.S., Harris, K.C., Dillmann, W.H., Cunningham, L.L., 2009. Hsp70 inhibits aminoglycoside-induced hearing loss and cochlear hair cell death. Cell Stress Chaperones 14, 427–437. doi:10.1007/s12192-008-0097-2

Thompson, A.M., Neely, J.G., 1992. Induction of heat shock protein in interdental cells by hyperthermia. Otolaryngol. Neck Surg. 107, 769–774. doi:10.1177/019459988910700611.1

WHO, 2017. Deafness and Hearing Loss [WWW Document]. WHO. URL http://www.who.int/mediacentre/factsheets/fs300/en/ (accessed 7.20.02).

Wroblewska-Seniuk, K.E., Dabrowski, P., Szyfter, W., Mazela, J., 2016. Universal newborn hearing screening: methods and results, obstacles, and benefits. Pediatr. Res. doi:10.1038/pr.2016.250

Xiao, X., Zuo, X., Davis, A. a., McMillan, D.R., Curry, B.B., Richardson, J. a., Benjamin, I.J., 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. EMBO J. 18, 5943–5952. doi:10.1093/emboj/18.21.5943

Yang, M., Tan, H., Yang, Q., Wang, F., Yao, H., Wei, Q., Tanguay, R.M., Wu, T., 2006. Association of hsp70 polymorphisms with risk of noise-induced hearing loss in Chinese automobile workers. Cell Stress Chaperones 11, 233–239. doi:10.1379/CSC-192R.1

Yoshida, N., Kristiansen, A., Liberman, M.C., 1999. Heat stress and protection from permanent acoustic injury in mice. J. Neurosci. 19, 10116–10124.

3.2. **Artigo 2. Plos One (research paper). Heat shock response in noise induced hearing loss: effects of alanyl-glutamine dipeptide supplementation on heat shock proteins status**

Heat shock response in noise-induced hearing loss: effects of alanyl-glutamine dipeptide supplementation on heat shock proteins status

Marcos Soares[1]*, Analu Bender dos Santos[1], Tainara Milbrandt Weich[1], Paulo Ivo Homem de Bittencourt Jr[2], Mirna Stela Ludwig[1] and Thiago Gomes Heck[1]*.

[1]Research Group in Physiology, Post Graduate Program in Integral Attention to Health (PPGAIS-UNIJUI/UNICRUZ), Department of Life Sciences, Regional University of Northwestern Rio Grande do Sul State (UNIJUI), Ijuí, RS, Brazil.

[2]Laboratory of Cellular Physiology, Department of Physiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

*corresponding author:

E-mail: marcossoaresorl@gmail.com (MS)

E-mail: thiago.heck@unijui.edu.br (TGH)

Abstract

The 72 kDa heat shock protein located intracellularly (iHSP72) has cochlear cytoprotective and anti-inflammatory roles in the inner ear during stressful noise challenges. The expression of iHSP72 can be potentiated by alanyl-glutamine dipeptide (DIP) supplementation. Conversely, these proteins act as pro-inflammatory signals in the extracellular *milieu.* We explore whether noise-induced hearing loss (NIHL) promotes both intracellular (iHSP72) and extracellular (eHSP72) heat shock response (HSR) alterations, and if DIP supplementation can modify HSR and prevent hearing loss. Female 90-day-old Wistar rats (n=32) were randomly divided into four groups: control (CON), noise-induced hearing loss (NIHL), treated with DIP (DIP), and NIHL plus DIP (DIP+NIHL). Auditory brainstem responses were evaluated before noise exposure (124 dB SPL for 2 h), and 14 days after. Cochlea, nuclear cochlear complex and plasma samples were collected for measurement of iHSP72 and eHSP72 by a high-sensitivity ELISA kit. The noise exposition induced an increase in auditory threshold, which was not prevented by DIP treatment. An increase in both iHSP72 and eHSP72 levels were observed in the NIHL group, which was alleviated by DIP treatment. Furthermore, H-index (plasma/cochlea eHSP72/iHSP72 ratio) was increased in the NIHL group, but prevented by DIP treatment. Our data indicates that cochlear damage induced by noise exposition is accompanied by local and systemic heat shock responses. Also, alanyl-glutamine supplementation failed to prevent noise-induced hearing loss, but did reduce stress markers; this warrants further investigation. Finally, plasma levels of 72 kDa heat shock proteins can be used as a biomarker of auditory stress, after noise exposure.

Introduction

Hearing loss affects approximately 360 million people worldwide, with a great impact on relationships and the ability to communicate. Although noise is the main evitable risk factor for hearing damage, it is estimated that 10% of the human population are exposed to excessive sound pressure, at levels that may induce auditory injury. Noise-induced hearing loss (NIHL) is the most prevalent occupational disease in the US, with 22 million workers exposed to high levels of noise, and requiring close to 240 million dollars in hearing loss treatment [1–3]. Many therapeutic strategies to treat or prevent NIHL have been investigated. Antioxidant therapies have shown success in preventing oxidative stress induced by noise exposition in animal models [3]. The Food and Drug Administration has recommended investigations into orally administered alternatives for hearing diseases [4,5].

A high level of noise exposition promotes intense metabolic activity in the cochlea, which induces oxidative stress associated with transient or permanent cochlear hair cell damage [4,6,7]. For protection against noise challenges, the cochlea requires a cytoprotective response, in the form of the expression of a family of 70 kDa heat shock proteins (HSP70). *HSPA1A* is the most studied heat shock response (HSR) gene, due to its high expression in mammalian cells under stress conditions. It is located at the major histocompatibility complex (MHC) III region, and encodes a 72 kDa inducible form (HSP72). In studies of heat-shocked preconditioned mice, the expression of these proteins increased, which provided protection against noise-induced hearing damage; this highlights the importance of HSP70 expression [8]. Also, suppression of heat shock factor 1 (HSF1), the transcription factor required for HSP72 synthesis, was shown to result in permanent hearing loss after noise exposition [9].

Several studies have assessed methods to induce the HSR in cochlea by assessing the cytoprotective role of HSP70 [10–14]. Glutamine is an amino acid that has been evaluated as a HSR potentiator, in both *in vitro* and *in vivo* studies [15,16]. The influence of glutamine or alanyl-glutamine dipeptide (DIP) on intracellular antioxidant defense due to increased glutathione levels [17], and the presence of glutamine transporters in cochlear hair cells, suggests that glutamine supplementation may be important for auditory health in subjects exposed to noise [18].

Intracellular HSP72 (iHSP72) acts as a molecular chaperone of other proteins (thereby limiting protein aggregation, facilitating protein refolding and maintaining structural function), and has anti-inflammatory properties through the inhibition of nuclear factor kB (NF-kB) activation [19]. On the other hand, increasing number of observations indicate that when located in extracellular *milieu* (eHSP72), these proteins can affect adjacent or distant cells [20,21]. eHSP72 is able to promote molecular interactions with cell surface receptors, and thus promote pro-inflammatory cell-signaling by interaction with a variety of eHSP72-receptors. In this respect, the release of eHSP72 to the extracellular *milieu* can be characterized as a pro-inflammatory state, whilst intracellular expression of iHSP72 represents a broader anti-inflammatory role. Based on these observations, the eHSP72/iHSP72 ratio (H-index) was established, where [eHSP72/iHSP70] at basal state is 1:1=1 (i.e., control group) represents a normal condition [22–24]. To our knowledge, the H-index has never used in hearing loss studies. In the present study using rats, we investigated whether noise exposition can induce HSR locally in the cochlea (iHSP72) and systemically (eHSP72). We also assessed whether DIP supplementation can modify HSR and prevent hearing loss. We hypothesized that plasma eHSP72 levels and/or the H-index can be used as biomarkers of auditory stress after noise exposure.

Materials and methods

Animals

Female 90-day-old Wistar rats (n=32) weighing approx. 200 g were obtained from Regional University of Northwestern Rio Grande do Sul State (UNIJUI) Animal Care Facility. They were maintained under a controlled temperature (23 ± 1°C) in a 12/12 h light/dark cycle (lights on at 07:00 a.m.), and housed in plastic cages (49 x 34 x 16 cm). Throughout the experiments, the rats had access to water and were fed with standard pelleted laboratory chow (NUVILAB CR-1, Nuvital Nutrients S.A., Curitiba, Brazil) *ad libitum*. The absence of otitis after specialist otoscopy was used as an inclusion criteria. Animals were randomly assigned into groups as described in the 'Experimental design' section. The investigation followed all ethical rules established by Arouca's Act (Federal Law 11794/2008) and the Guide for Care and Use of Experimental Animals, published by the National Institutes of Health (NIH publication no. 85–23, revised in 1996). All procedures were approved by the Committee of Animal Welfare Regional University of Northwestern Rio Grande do Sul State (CEUA-UNIJUI, protocol #058/15).

Experimental design

For seven consecutive days, the rats received oral alanyl-glutamine dipeptide (DIP) or water (vehicle), and were then evaluated by the auditory brainstem response test (ABR). One day later, half of the animals were exposed to noise for 2 h, and then all (n=32) were split into the following experimental groups (n=8 per group): Control (CON), noise-induced hearing loss (NIHL), treated with DIP (DIP), and NIHL plus DIP

treatment (DIP+NIHL). The DIP and DIP+NIHL groups were treated with DIP for more than fourteen days after noise exposition. The ABR test was repeated in all rats, and cochlea and nuclear cochlear complexes were surgery extracted by a specialist. Plasma samples were also obtained for analyses. A summary of the experimental design is shown in Fig 1.

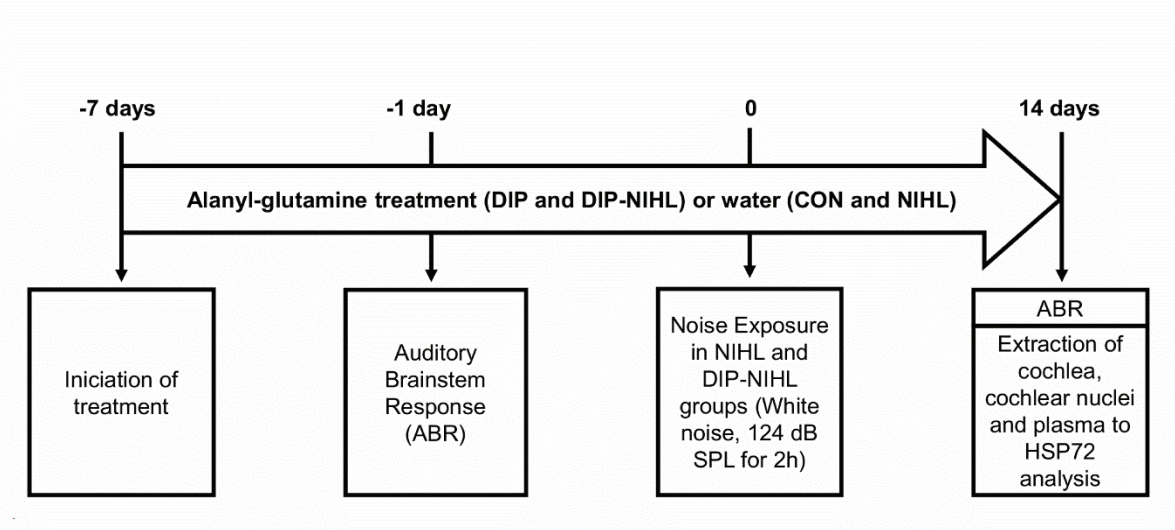

Fig 1. Experimental design. Seven days before the induction of the animal model of noise-induced hearing loss, treatment with alanyl-glutamine (DIP and DIP-NIHL groups) or water (CON and NIHL groups) was initiated. One day before, auditory evaluation with ABR was performed to determine basal hearing thresholds. At day zero, the NIHL and DIP-NIHL groups were exposed to 124 dB SPL for 2 h. After 14 days, ABR was performed in all animals, followed by extraction of the cochlea, cochlear nuclei, and plasma for measurement of HSP72 concentration.

Alanyl-glutamine dipeptide (DIP) supplementation

The rats were supplemented daily with L-alanyl-L-glutamine DIP (Dipeptiven®, Fresenius Kabi®) at a dose of 1.5 g/kg (diluted in water to a final concentration of 0.2 g/mL). The animals received supplements through gavage feeding (1 mL/100 g bodyweight) for 21 days (7 days before, and 14 days after, noise exposition).

Auditory brainstem response (ABR)

For the auditory evaluation of the rats, auditory brainstem response (ABR) was performed using the Vivosonic Integrity V500 system®. The rats were anesthetized intraperitoneally with ketamine (80 mg/kg) and xylazine (10 mg/kg), and placed in an anechoic room. Subcutaneous needle-type electrodes were inserted posterior to the tested pinna (active electrode), vertex (reference electrode), and contralateral pinna (ground electrode). The sound stimuli were clicks (rise/fall time, 2 ms; total duration, 2 ms; repetition rate, 21/s). The responses were filtered (100–3000 Hz bandpass), and averaged across 500 samples.

The rat ABR consists of four components (labeled P1 to P4) which occur within 6 ms of the stimulus onset. These components reflect the neural activity of the auditory nerve (P1), the cochlear nucleus (P2), the superior olivary complex (P3), and the lateral lemniscus and/or inferior colliculus (P4). Hearing thresholds were determined by decreasing the sound intensity in 5 dB steps, starting at 100 dB and decreasing to 0 dB, or until a reliably-scored ABR component was detected. In rodents, the ABR P2 wave is the largest and usually the last to disappear as the sound stimulus decreases. Hence, the threshold value was defined as the lowest intensity able to elicit a P2 wave [25], and ABR data was expressed as hearing thresholds (HT), and hearing threshold shifts (HTS). The latter represents the difference between the thresholds, before and after the noise exposure of each rat.

Noise exposure

The rats were exposed for 2 h to continuous white noise of a broad spectrum of frequency and a peak intensity of 8000 Hz, at 124 dB SPL. During the exposure, the

rats were placed in a box inside an anechoic room. White noise was produced by an audio signal generator, connected to speakers in the center of the box (EP125, Insight®). The noise level was measured using a decibelimeter (TDEC100 Digital Decibelimeter, Incoterm®) located inside the box at the start, and after completion, of the noise exposure.

Cochlea, and cochlear nuclear complex iHSP72 and plasma eHSP72 levels

Cochlea (two for each animal) and nuclear cochlear complex samples were quickly removed and washed in ice-cold phosphate-buffered saline (PBS), pH 7.4. Samples were homogenized mechanically inside microtubes containing 50 uL of potassium phosphate buffer with protease inhibitor (phenyl-methyl-sulfonyl fluoride, PMSF, 100 μM) and centrifuged (5000 rpm for 10 min). The supernatants were frozen in microtubes containing liquid nitrogen, until analysis of iHSP70 levels. Plasma was obtained by centrifugation (blood with EDTA; 3000 rpm for 10 min) and stored at -20°C for analysis of eHSP72 levels. Cochlea and nuclear cochlear complex iHSP70 levels and plasma eHSP70 levels were measured using a high sensitivity ELISA kit (AMP'D® HSP70 high sensitivity ELISA kit, EnzoLifesciences®).

Extracellular-to-intracellular HSP70 ratio index (H-index)

Extracellular-to-intracellular HSP70 ratio index (H-index) has been described as a novel and overall index of the immunoinflammatory status of an individual [19,26,27,23,24,22]. The rationale for H-index is that the higher the level of eHSP70, the greater the inflammatory signal, due to the pro-inflammatory nature of the protein. Conversely, if cells are able to respond to stressful stimuli by enhancing iHSP70

production, they simultaneously enter a state of anti-inflammation. Therefore, if Rc=[eHSP70]c/[iHSP70]c represents the HSP70 ratio in a controlled situation, the H-index for a stressful situation (Rj) can be calculated as the quotient of different values of Rj=[eHSPA]j/[iHSPA]j, relative to Rc (where Rc=1 i.e., baseline). Hence, the H-index (Rj/Rc) allows comparisons between any stressful situation and the control [22].

Statistical analysis

Preceding the statistical analysis, all outcome variables were assessed for normality using the Kolmogorov-Smirnov test. Data is presented as the mean ± S.D. For the analysis of hearing loss and HSR response, the minimum sample size required to detect differences (keeping $\alpha=0.05$ and test power of 80%), is 8 rats in each group [28]. The t-test was used to compare basal and final hearing thresholds within groups. Comparisons between groups were performed by one-way ANOVA followed by Student-Newman-Keuls post-hoc test.

Results

Before any intervention, all the rats showed an auditory threshold close to 20 dB (Fig 2a). Noise exposition promoted a 40 dB increase in auditory threshold in the NIHL and the DIP+NIHL groups (Fig 2a-b).

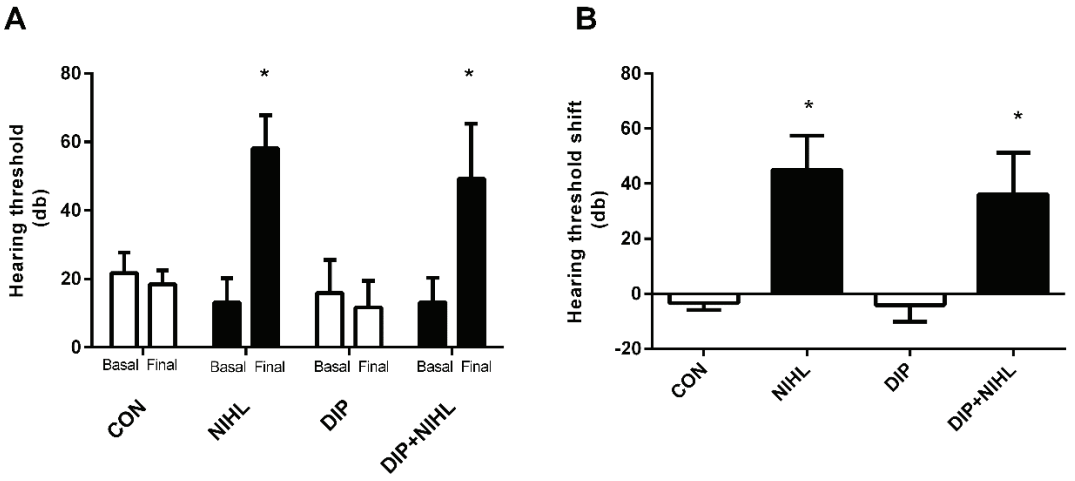

Fig 2. Effects of alanyl-glutamine dipeptide (DIP) treatment on noise-induced hearing loss (NIHL). (a) Hearing threshold and (b) Hearing threshold shift. NIHL and DIP+NIHL showed an increase in hearing threshold (* p<0.05 vs same group before noise exposure) and hearing threshold shift (** P<0.05 vs groups without noise exposure).

Noise exposition (14 days) induced an increase in cochlear iHSP70 levels (NIHL group) when compared to the control group (Fig 3a), whilst iHSP70 levels in the nuclear cochlear complex were unaltered (Fig 3b). The eHSP72 levels increased due to noise exposition, and this effect was blunted by DIP treatment (Fig 3c).

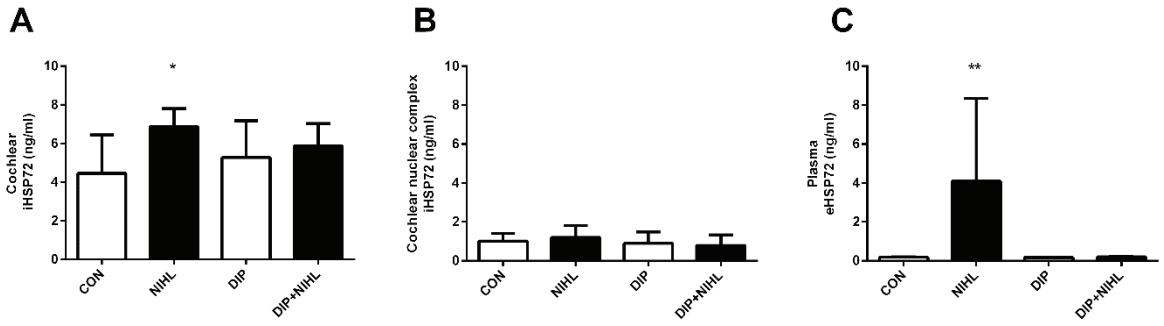

Fig 3. Effects of alanyl-glutamine dipeptide (DIP) treatment on noise-induced hearing loss (NIHL) heat shock response (HSR). NIHL promoted an increase in cochlear iHSP72 expression (* $p<0.05$ vs control) (a). No changes in iHSP72 expression were observed in cochlear nuclear complex (b). Plasma eHSP72 concentrations were higher in the NIHL group than all groups combined (** $p<0.05$) (c).

After noise exposition, the eHSP70/iHSP70 ratio heat shock status (H-index) was evaluated. Increases were observed in both plasma/cochlear HSP70 ratio (Fig 4a) and plasma/nuclear cochlear complex HSP70 ratio (Fig 4b). Treatment with DIP removed these effects and resulted in H-index levels, in plasma/cochlear and plasma/nuclear cochlear complex, that were similar to the control group.

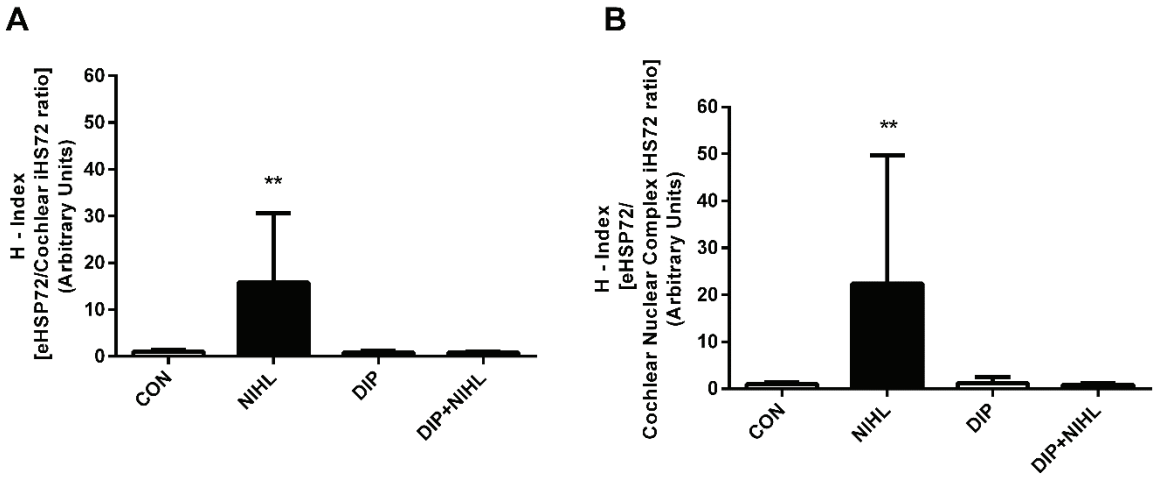

Fig 4. Effects of alanyl-glutamine dipeptide (DIP) treatment on noise-induced hearing loss (NIHL) eHSP72/iHSP72 ratio (H-index). (a) plasma/cochlear HSP70 ratio and (b) plasma/nuclear cochlear complex HSP70 ratio. NIHL promoted an increase in both plasma/cochlear and plasma/cochlear nuclear complex eHSP72/iHSP72 ratio (* $p<0.05$ vs all groups).

Discussion

This study reports the first evidence that cochlear damage induced by noise exposition is accompanied by local and systemic HSR, and DIP supplementation can

attenuate stress markers in a NIHL rat model. We also observed that DIP treatment did not prevent hearing loss, despite the decreases in stress marker levels.

The rat model of NIHL was based on a previous study, in which loss of hearing was induced using 124 dB SPL for 2 h [28]. This differed from our study in the use of Sprague-Dawley rats, and peak sound intensity set at a frequency of 4000 Hz. The present study also used a click stimulus for ABR. Both click and pure-tone stimuli are commonly used in investigations of this nature. The click stimulus has high reproducibility and waveform stability, and is one of the most common stimuli used in clinical studies. In humans, auditory evaluation using clicks produces a frequency spectrum of 2000–4000 Hz, compared to 8000-10000 Hz in rats. A possible explanation for this difference is the anatomical differences of human and rat ears [29]. For our study, click stimulus was suitable for cochlear evaluation because of its excellent reproducibility. Due to the spectral matching (8000–10000 Hz) of the click stimulus, a peak sound intensity of 124 dB SPL was chosen at 8000 Hz. Even with the differences between these studies, both sets of results demonstrated similar hearing threshold shifts of approximately 40 dB, 14 days after noise exposure.

Tissue HSR is required for the maintenance of a balanced inflammatory status, due to the cytoprotective and anti-inflammatory roles of iHSP70 expression. This is essential for proteostasis against harmful challenges such as oxidative stress [30]. Several stressors are able to induce iHSP70 expression in the cochlea of rodents, including whole body heat-shock [8], local hyperthermia [31], transitory isquemia [32], cisplatin ototoxicity [33], and high levels of noise exposition [8,10,34]. The role of iHSP70 in situations of noise stress was investigated by Fairfield et al. [9] and Gong et al.[35]. Both groups showed the importance of heat shock factor 1 (HSF1), the main transcription factor of HSP70 family, in cochlear damage prevention and repair after

intense noise exposition in knockout hsf-/-mice [9,35]. HSR initiated soon after noise exposure (106 dB NPS for 2 h), with a peak of HSP70 mRNA expression after 4 h [35]. We have demonstrated, for the first time, a persistent stress response of the auditory system after a noise challenge. Rats from the NIHL group exhibited sustained increases in iHSP70 levels for fourteen days after noise exposure. However, DIP treatment did not result in potentiation of iHSP70 in cochlear or cochlear nuclear complex, and so failed to prevent hearing loss, i.e., the posology (dose, frequency and period of treatment) of DIP did not lead to improvements in HSR compared to the control rats. The failure to initiate a robust HSR under stressful situations is a serious impairment of cell function [22,36].

The InterAcademy Medical Panel recommends the avoidance and treatment of NIHL [1]. Accordingly, we investigated the effect of DIP supplementation on hearing loss. L-glutamine is the most abundant free amino acid in the body, nutritionally classified as a nonessential amino acid. Since glutamine is the immediate precursor of glutamate, *de novo* synthesis of glutathione may increase with DIP treatment, and provide an additional antioxidant defense in intracellular spaces [17,37], and thus prevent oxidative stress damage [38]. These effects have been observed in studies that administered the same DIP doses (1.5 g/kg) used in our study [39,40]. Only a few studies have investigated glutamine cochlear metabolism. Ryan and Schwartz [41] traced glutamine uptake into cochlear hair cells, and observed higher levels in the inner hair cells than those of the outer cells. This suggests the existence of a membrane high-affinity system for glutamine transport in cochlea. In addition, glutamate is the main neurotransmitter used by cochlea auditory signaling, mainly by inner hair cells. Consequently, these cells are rich in glutaminase enzymes [42]. Dendritic presynaptic membranes express high amounts of α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptor (AMPA) glutamate receptor SAT1 (alternatively termed GlnT, SA2, SNAT1 and ATA1), a member of the neutral amino acids transporter family (SLC38). It has preferential affinity for glutamine, and transports this amino acid from the endolymph (extracellular space) to inner hair cells for glutamate synthesis [18].

The protective effects of glutamine are associated with HSR potentiation, as it can promote a slight increase in heat shock factor 1 (HSF-1) trimerization, a step required for HSR. However, under challenge situations such as heat shock, glutamine increases HSF1 activation, and consequently HSP70 synthesis [37]. Activation of the O-linked glycosylation (O-GlcNAc) pathway by glutamine can promote reciprocal phosphorylation [15], which leads to nuclear translocation of HSF1 to the heat shock element (HSE) region that is responsible for HSP70 family expression [16].

These factors strengthens the hypothesis that DIP supplementation is beneficial to the auditory system, with roles in signaling, antioxidant and cell stress defense. Although DIP supplementation (at 1.5 g/kg) can promote a 62% increase in plasma glutamine concentration [17], the DIP dose required to improve cochlear cell function and protection is unclear [38]. Additionally, the time-course of cochlear HSR under noise stress requires further investigation, especially since rapid HSR was observed after 4 h and maintained for the fourteen days of the trial.

A crucial aspect of HSP70 physiology is the versatility and duality of these proteins. iHSP70 acts as anti-inflammatory agent by inhibiting the activation of nuclear transcription factors of the kappa light chain enhancer of activated B cells (κB, family; NF-κB) at multiple regulatory levels [37]. In contrast, eHSP70 has an opposite function. It signals the presence of homeostatic challenges to physiological systems, after binding to Toll-like receptors (TLR-2, -4, and -7) in a variety of cells [19]. This leads to

the activation of pro-inflammatory pathways [43]. Since the discovery of eHSP70s in the circulatory system, many associations between eHSP70 levels and bad prognosis in patients have been described in several diseases, usually related to oxidative stress. Release of eHSP70 occurs through several pathways. It is thought that HSP70 is found in plasma as a result of an active process such as danger signaling, or by passive necrotic cell death [21]. For the first time, we have demonstrated that the harmful effects of noise exposition can be detected by an increase in eHSP72 plasma levels. Furthermore, the decrease in eHSP70 may facilitate the development of DIP-based therapy for the treatment of hearing loss.

We evaluated cochlea and nuclear cochlear complex iHSP70 and plasma eHSP70 levels using a high sensitivity ELISA kit. It was assumed that both the inducible HSPA1A and HSPA6 (HSP70B') forms, as well as the cognate HSPA8 form of HSP70, would accumulate in the extracellular space of different cell types after appropriate stressful stimuli. However, only the HSPA1A ELISA kit has undergone global evaluation, and has the sensitivity (pg/mL range) to detect minute HSP70 quantities in culture media and sera. Additionally, previous results of this laboratory [23] have indicated that the principal eHSP70 forms (HSPA1A and HSPA8) are secreted in similar amounts. Therefore, it was surmised that HSPA1A is representative of the total eHSP70 secretion. The results of the present study indicate that cochlear damage induced by noise exposition is accompanied by local and systemic HSR. Thus, plasma levels of eHSP72 can be used as a biomarker of cochlear stress due to noise exposure.

Due to the versatility of HSP70 in inducing different inflammation responses according to its location, it is likely that this protein may be an important marker for immunoinflammatory state during exercise [26]. Indeed, its level in circulation appears

to be fundamental to the maintenance of homeostasis [19]. In addition, the [eHSP72/iHSP70] ratio balance, measured by the mathematical calculation H-index, may represent an important biomarker of health, and serve as a reference for subclinical biological processes [25–27].

Our data demonstrates that DIP treatment blunts the release of eHSP72 in noise-exposed rats. Possibly, glutamine decreases oxidative stress and thereby reduces cellular stress. We can discard the hypothesis that decreases in eHSP72 release is due to cellular malfunction (e.g., cell death, necrosis or apoptosis), since the auditory threshold was similar in the NIHL and DIP+NIHL groups. In addition, the H-index mean in the NIHL group was 16.0, whilst in the DIP+NIHL group it was approximately 1.0 (arbitrary units of eHSP72/iHSP72 concentration). These findings are satisfactory because H-index may stratify HSP70 status (i.e., H-index of one, relative to the normal profile, and higher H-indices under a stress profile), as well as other clinical conditions according to their immunoinflammatory states [22]. Indeed, the H-index has recently emerged as a potential biomarker of the effect of stressful situations on the immune system, and of immunoinflammatory imbalances related to cytokine fluctuations and poor HSR. Finally, the high H-Index value observed herein (15.7 in NIHL group) is approaching the inflection point (19.18, arbitrary units of eHSP72/iHSP70 ratio), which represents a dangerous pro-inflammatory profile [22].

Conclusion

Our data indicates that cochlear damage induced by noise exposition is accompanied by local and systemic heat shock responses. Also, DIP supplementation did not prevent noise-induced hearing loss, but did promote a reduction in stress markers; this warrants further investigation. The results of this study confirm that

plasma levels of 72 kDa heat shock proteins can be used as biomarkers of auditory stress after noise exposure.

Acknowledgements

The authors would like to thank M. Turcato (UNIJUÍ), and colleagues from the Research Group in Physiology (UNIJUI) for their technical support.

References

1. Looi LM, Ganten D, McGrath PF, Gross M, Griffin GE. Hearing loss: a global health issue. Lancet. 2015;385: 943–944. doi:10.1016/S0140-6736(15)60208-2

2. World Health Organization. Community-based rehabilitation promoting ear and hearing care through CBR. 2012; 28.

3. Basner M, Babisch W, Davis A, Brink M, Clark C, Janssen S, et al. Auditory and non-auditory effects of noise on health. Lancet. 2014;383: 1325–1332. doi:10.1016/S0140-6736(13)61613-X

4. Bao J, Hungerford M, Luxmore R, Ding D, Qiu Z, Lei D, et al. Prophylactic and therapeutic functions of drug combinations against noise-induced hearing loss. Hear Res. 2013;304: 33–40. doi:10.1016/j.heares.2013.06.004

5. Mukherjea D, Ghosh S, Bhatta P, Sheth S, Tupal S, Borse V, et al. Early investigational drugs for hearing loss. Expert Opin Investig Drugs. 2014;24: 1–17. doi:10.1517/13543784.2015.960076

6. Henderson D, Bielefeld EC, Harris KC, Hu BH. The role of oxidative stress in

noise-induced hearing loss. Ear Hear. 2006;27: 1–19. doi:10.1097/01.aud.0000191942.36672.f3

7. Chen Z, Ulfendahl M, Ruan R, Tan L, Duan M. Protection of auditory function against noise trauma with local caroverine administration in guinea pigs. Hear Res. 2004;197: 131–136. doi:10.1016/j.heares.2004.03.021

8. Yoshida N, Kristiansen A, Liberman MC. Heat stress and protection from permanent acoustic injury in mice. J Neurosci. 1999;19: 10116–10124.

9. Fairfield D a., Lomax MI, Dootz G a., Chen S, Galecki AT, Benjamin IJ, et al. Heat shock factor 1-deficient mice exhibit decreased recovery of hearing following noise overstimulation. J Neurosci Res. 2005;81: 589–596. doi:10.1002/jnr.20417

10. Lim HH, Jenkins OH, Myers MW, Miller JM, Altschuler R a. Detection of HSP 72 synthesis after acoustic overstimulation in rat cochlea. Hear Res. 1993;69: 146–150. doi:10.1016/0378-5955(93)90102-7

11. Cunningham LL, Brandon CS. Heat shock inhibits both aminoglycoside- and cisplatin-induced sensory hair cell death. J Assoc Res Otolaryngol. 2006;7: 299–307. doi:10.1007/s10162-006-0043-x

12. Baker TG, Roy S, Brandon CS, Kramarenko IK, Francis SP, Taleb M, et al. Heat shock protein-mediated protection against cisplatin-induced hair cell death. J Assoc Res Otolaryngol. 2014;16: 67–80. doi:10.1007/s10162-014-0491-7

13. Taleb M, Brandon CS, Lee FS, Harris KC, Dillmann WH, Cunningham LL.

Hsp70 inhibits aminoglycoside-induced hearing loss and cochlear hair cell death. Cell Stress Chaperones. 2009;14: 427–437. doi:10.1007/s12192-008-0097-2

14. Liu Y, Yu Y, Chu H, Bing D, Wang S, Zhou L, et al. 17-DMAG induces Hsp70 and protects the auditory hair cells from kanamycin ototoxicity in vitro. Neurosci Lett. 2015;588: 72–77. doi:10.1016/j.neulet.2014.12.060

15. Hamiel CR, Pinto S, Hau A, Wischmeyer PE. Glutamine enhances heat shock protein 70 expression via increased hexosamine biosynthetic pathway activity. Am J Physiol Cell Physiol. 2009;297:1509–1519. doi:10.1152/ajpcell.00240.2009

16. Singleton KD, Wischmeyer PE. Glutamine's protection against sepsis and lung injury is dependent on heat shock protein 70 expression. Am J Physiol Regul Integr Comp Physiol. 2007;292: 1839–1845. doi:10.1152/ajpregu.00755.2006

17. Petry ÉR, Cruzat VF, Heck TG, Leite JSM, Homem de Bittencourt PI, Tirapegui J. Alanyl-glutamine and glutamine plus alanine supplements improve skeletal redox status in trained rats: Involvement of heat shock protein pathways. Life Sci. 2014;94: 130–136. doi:10.1016/j.lfs.2013.11.009

18. Oguchi T, Suzuki N, Hashimoto S, Chaudhry GA, Chaudhry FA, Usami SI, et al. Inner hair cells of mice express the glutamine transporter SAT1. Hear Res. 2012;292: 59–63. doi:10.1016/j.heares.2012.07.005

19. Heck TG, Schöler CM, de Bittencourt PIH. HSP70 expression: Does it a novel fatigue signalling factor from immune system to the brain? Cell Biochem Funct. 2011;29: 215–226. doi:10.1002/cbf.1739

20. Krause M, Rodrigues-Krause JDC. Extracellular heat shock proteins (eHSP70) in exercise: Possible targets outside the immune system and their role for neurodegenerative disorders treatment. Med Hypotheses. 2011;76: 286–290. doi:10.1016/j.mehy.2010.10.025

21. De Maio A. Extracellular Hsp70: export and function. Curr Protein Pept Sci. 2014;15: 225–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24694368

22. Heck TG, Scomazzon SP, Nunes PR, Schöler CM, da Silva GS, Bittencourt A, et al. Acute exercise boosts cell proliferation and the heat shock response in lymphocytes: correlation with cytokine production and extracellular-to-intracellular HSP70 ratio. Cell Stress Chaperones. 2017;22: 271–291. doi:10.1007/s12192-017-0771-3

23. Schöler CM, Marques CV, da Silva GS, Heck TG, de Oliveira Junior LP, Homem de Bittencourt PI. Modulation of rat monocyte/macrophage innate functions by increasing intensities of swimming exercise is associated with heat shock protein status. Mol Cell Biochem. 2016;421: 111–25. doi:10.1007/s11010-016-2791-1

24. Goettems-Fiorin PB, Grochanke BS, Baldissera FG, dos Santos AB, Homem de Bittencourt PI, Ludwig MS, et al. Fine particulate matter potentiates type 2 diabetes development in high-fat diet-treated mice: stress response and extracellular to intracellular HSP70 ratio analysis. J Physiol Biochem. 2016;72:643-56. doi:10.1007/s13105-016-0503-7

25. Fetoni AR, De Bartolo P, Eramo SLM, Rolesi R, Paciello F, Bergamini C, et al. Noise-induced hearing loss (NIHL) as a target of oxidative stress-mediated

damage: cochlear and cortical responses after an increase in antioxidant defense. J Neurosci. 2013;33: 4011–23. doi:10.1523/JNEUROSCI.2282-12.2013

26. Krause M, Heck TG, Bittencourt A, Scomazzon SP, Newsholme P, Curi R, et al. The chaperone balance hypothesis : the importance of the extracellular to intracellular HSP70 ratio to inflammation-driven type 2 diabetes , the effect of exercise , and the implications for clinical management. Mediators Inflamm. 2015;2015:12.

27. Krause M, Ludwig MS, Heck TG, Takahashi HK. Heat shock proteins and heat therapy for type 2 diabetes: pros and cons. Curr Opin Clin Nutr Metab Care. 2015;18: 374–80. doi:10.1097/MCO.0000000000000183

28. Fujioka M, Kanzaki S, Okano HJ, Masuda M, Ogawa K, Okano H. Proinflammatory cytokines expression in noise-induced damaged cochlea. J Neurosci Res. 2006;83: 575–83. doi:10.1002/jnr.20764

29. Sanz-Fernández R, Sánchez-Rodriguez C, Granizo JJ, Durio-Calero E, Martín-Sanz E. Utility of auditory steady-state and brainstem responses in age-related hearing loss in rats. Acta Otolaryngol. 2015;135: 35–41. doi:10.3109/00016489.2014.953203

30. Newsholme P, de Bittencourt PIH. The fat cell senescence hypothesis: a mechanism responsible for abrogating the resolution of inflammation in chronic disease. Curr Opin Clin Nutr Metab Care. 2014;17: 295–305. doi:10.1097/MCO.0000000000000077

31. Sugahara K, Inouye S, Izu H, Katoh Y, Katsuki K, Takemoto T, et al. Heat

shock transcription factor HSF1 is required for survival of sensory hair cells against acoustic overexposure. Hear Res. 2003;182: 88–96. doi:10.1016/S0378-5955(03)00180-1

32. Myers MW, Quirk WS, Rizk SS, Miller JM, Altschuler RA. Expression of the major mammalian stress protein in the rat cochlea following transient ischemia. Laryngoscope. 1992;102: 981–987. doi:10.1288/00005537-199209000-00005

33. Oh SH, Yu WS, Song BH, Lim D, Koo JW, Chang SO, et al. Expression of heat shock protein 72 in rat cochlea with cisplatin-induced acute ototoxicity. Acta Otolaryngol. 2000;120: 146–150. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11603760

34. Yoshida N, Liberman MC. Sound conditioning reduces noise-induced permanent threshold shift in mice. Hear Res. 2000;148: 213–219. doi:10.1016/S0378-5955(00)00161-1

35. Gong TW, Fairfield D a., Fullarton L, Dolan DF, Altschuler R a., Kohrman DC, et al. Induction of heat shock proteins by hyperthermia and noise overstimulation in Hsf1-/- mice. J Assoc Res Otolaryngol. 2012;13: 29–37. doi:10.1007/s10162-011-0289-9

36. Török Z, Crul T, Maresca B, Schütz GJ, Viana F, Dindia L, et al. Plasma membranes as heat stress sensors: from lipid-controlled molecular switches to therapeutic applications. Biochim Biophys Acta. 2014;1838: 1594–618. doi:10.1016/j.bbamem.2013.12.015

37. Leite JSM, Cruzat VF, Krause M, Homem de Bittencourt PI. Physiological regulation of the heat shock response by glutamine: implications for chronic

low-grade inflammatory diseases in age-related conditions. Nutrire. 2016;41: 17. doi:10.1186/s41110-016-0021-y

38. de Oliveira DC, da Silva Lima F, Sartori T, Santos ACA, Rogero MM, Fock RA. Glutamine metabolism and its effects on immune response: molecular mechanism and gene expression. Nutrire. 2016;41: 14. doi:10.1186/s41110-016-0016-8

39. Rogero MM, Tirapegui J, Pedrosa RG, de Castro IA, de Oliveira Pires IS. Effect of alanyl-glutamine supplementation on plasma and tissue glutamine concentrations in rats submitted to exhaustive exercise. Nutrition. 2006;22: 564–571. doi:10.1016/j.nut.2005.11.002

40. Cruzat VF, Bittencourt A, Scomazzon SP, Leite JSM, De Bittencourt PIH, Tirapegui J. Oral free and dipeptide forms of glutamine supplementation attenuate oxidative stress and inflammation induced by endotoxemia. Nutrition. 2014;30: 602–611. doi:10.1016/j.nut.2013.10.019

41. Ryan AF, Schwartz IR. Preferential glutamine uptake by cochlear hair cells: implications for the afferent cochlear transmitter. Brain Res. 1984;290: 376–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6140989

42. Nordang L, Cestreicher E, Arnold W, Anniko M. Glutamate is the afferent neurotransmitter in the human cochlea. Acta Otolaryngol. 2000;120: 359–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10894409

43. De Maio A. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. It is never known how far a controversial finding will go!

Dedicated to Ferruccio Ritossa. Cell Stress Chaperones. 2011;16: 235–49. doi:10.1007/s12192-010-0236-4

Financial support

This work was supported by grants from the Research Support Foundation of the State of Rio Grande do Sul (PqG-2013-FAPERGS process 002106-2551/13-5, and ARD/PPP/FAPERGS/CNPq-08/2014 process 16/2551-0000196-6), and by CNPq (UNIVERSAL MCTI/CNPq Nº 01/2016). ABS was a recipient of a scholarship from the Coordination for the Improvement of Higher Education Personnel (CAPES).

Disclosure of interest

The authors report no conflicts of interest and no competing interests such as substances, financial involvement or patent ownership, relating to this study.

4. CONSIDERAÇÕES FINAIS

O uso da alanilglutamina, na dose utilizada, não foi capaz de prevenir uma perda auditiva induzida pelo ruído. Por outro lado, foi evidenciada liberação de eHSP72 plasmática na PAIR, fato ainda não demonstrado em nenhum estudo. A análise do estado inflamatório global, pelo cálculo do índice-H, evidenciou altos índices no grupo PAIR. Em contraponto, o tratamento com alanilglutamina diminuiu a concentração de eHSP72 no plasma, mantendo os níveis do índice-H semelhantes ao do controle, demonstrando potencial anti-inflamátório.

O papel citoprotetor das iHSP70 coclear já está solidificado. A pesquisa de substâncias ou condições indutoras destas proteínas na cóclea é um caminho promissor para prevenção e/ou tratamento das patologias que causem dano na orelha interna. Estudos que investiguem o papel das eHSP70 na fisiologia coclear podem trazer novas abordagens terapêuticas ou explicar novos mecanismos fisiopatológicos de fatores que causam perda auditiva.

Como perspectiva, são necessários novos estudos para evidenciar se doses mais elevadas de alanilglutamina podem proteger a audição frente a um ruído, além de investigar mecanismos na cóclea relacionadas a expressão de iHSP72.

5. REFERÊNCIAS BIBLIOGRÁFICAS

ASEA, A. Novel Signal Transduction Pathway Utilized by Extracellular HSP70. ROLE OF Toll-LIKE RECEPTOR (TLR) 2 AND TLR4. **Journal of Biological Chemistry**, v. 277, n. 17, p. 15028–15034, 2002.

BAKER, T. G. et al. Heat Shock Protein-Mediated Protection Against Cisplatin-Induced Hair Cell Death. **Journal of the Association for Research in Otolaryngology**, v. 16, n. 1, p. 67–80, 2014.

BAO, J. et al. Prophylactic and therapeutic functions of drug combinations against noise-induced hearing loss. **Hearing Research**, v. 304, p. 33–40, 2013.

BARBE, M. F. et al. Hyperthermia protects against light damage in the rat retina. **Science (New York, N.Y.)**, v. 241, n. 4874, p. 1817–20, 30 set. 1988.

BASNER, M. et al. Auditory and non-auditory effects of noise on health. **The Lancet**, v. 383, p. 1325–1332, 2014.

BOBKOVA, N. V et al. Therapeutic effect of exogenous hsp70 in mouse models of Alzheimer's disease. **Journal of Alzheimer's disease : JAD**, v. 38, n. 2, p. 425–35, 2014.

BOHNE, B. A.; ZAHN, S. J.; BOZZAY, D. G. Damage to the Cochlea following Interrupted Exposure to Low Frequency Noise. **Annals of Otology, Rhinology & Laryngology**, v. 94, n. 2, p. 122–128, mar. 1985.

BORGES, T. J. et al. The anti-inflammatory mechanisms of Hsp70. **Frontiers in Immunology**, v. 3, n. MAY, p. 1–12, 2012.

CANLON, B.; BORG, E.; FLOCK, A. Protection against noise trauma by pre-exposure to a low level acoustic stimulus. **Hearing research**, v. 34, n. 2, p. 197–200, 15 jul. 1988.

CIORBA, A. et al. The impact of hearing loss on the quality of elderly adults. **Clinical Interventions in Aging**, n. 7, p. 159–163, 2012.

CRUZAT, V. F. et al. Oral free and dipeptide forms of glutamine supplementation

attenuate oxidative stress and inflammation induced by endotoxemia. **Nutrition**, v. 30, n. 5, p. 602–611, 2014.

CUNNINGHAM, L. L.; BRANDON, C. S. Heat shock inhibits both aminoglycoside- and cisplatin-induced sensory hair cell death. **JARO - Journal of the Association for Research in Otolaryngology**, v. 7, p. 299–307, 2006.

DANIEL, E. Noise and hearing loss: A review. **Journal of School Health**, v. 77, n. 5, p. 225–231, 2007.

DE MAIO, A. Extracellular Hsp70: export and function. **Current protein & peptide science**, v. 15, n. 3, p. 225–31, 2014.

DECHESNE, C. J. et al. Expression of heat shock protein, HSP72, in the guinea pig and rat cochlea after hyperthermia: Immunochemical and in situ hybridization analysis. **Hearing Research**, v. 59, n. 2, p. 195–204, 1992.

EKIMOVA, I. V et al. Exogenous protein Hsp70/Hsc70 can penetrate into brain structures and attenuate the severity of chemically-induced seizures. **Journal of neurochemistry**, v. 115, n. 4, p. 1035–44, 2010.

FAIRFIELD, D. A. et al. Expression and localization of heat shock factor (Hsf) 1 in the rodent cochlea. **Hearing Research**, v. 173, n. 1–2, p. 109–118, 2002.

FAIRFIELD, D. A. et al. Heat shock factor 1-deficient mice exhibit decreased recovery of hearing following noise overstimulation. **Journal of Neuroscience Research**, v. 81, p. 589–596, 2005.

FRANCIS, S. P. et al. Celastrol inhibits aminoglycoside-induced ototoxicity via heat shock protein 32. **Cell death & disease**, v. 2, n. 8, p. e195, 2011.

FUJIOKA, M. et al. Proinflammatory cytokines expression in noise-induced damaged cochlea. **Journal of neuroscience research**, v. 83, n. 4, p. 575–83, mar. 2006.

GOETTEMS-FIORIN, P. B. et al. Fine particulate matter potentiates type 2 diabetes development in high-fat diet-treated mice: stress response and extracellular to intracellular HSP70 ratio analysis. **Journal of Physiology and Biochemistry**, 2016.

GONG, T. W. et al. Induction of heat shock proteins by hyperthermia and noise overstimulation in Hsf1?/? mice. **JARO - Journal of the Association for Research in Otolaryngology**, v. 13, p. 29–37, 2012.

HAMIEL, C. R. et al. Glutamine enhances heat shock protein 70 expression via increased hexosamine biosynthetic pathway activity. **American journal of physiology. Cell physiology**, v. 297, n. 6, p. C1509–C1519, 2009.

HECK, T. G. Razão entre o conteúdo extracelular e intracelular de HSP70 como um sinal de alerta imunológico e marcador de intensidade de exercício. p. 226, 2011.

HECK, T. G. et al. Acute exercise boosts cell proliferation and the heat shock response in lymphocytes: correlation with cytokine production and extracellular-to-intracellular HSP70 ratio. **Cell Stress and Chaperones**, p. 1–21, 1 mar. 2017.

HECK, T. G.; SCHÖLER, C. M.; DE BITTENCOURT, P. I. H. HSP70 expression: Does it a novel fatigue signalling factor from immune system to the brain? **Cell Biochemistry and Function**, v. 29, n. January, p. 215–226, 2011.

HENDERSON, B. Integrating the cell stress response: a new view of molecular chaperones as immunological and physiological homeostatic regulators. **Cell Biochemistry and Function**, v. 28, n. 1, p. 1–14, jan. 2010.

HENDERSON, D. et al. The role of oxidative stress in noise-induced hearing loss. **Ear and hearing**, v. 27, n. 1, p. 1–19, 2006.

JIANG, H.; SHA, S.-H.; SCHACHT, J. NF-?B pathway protects cochlear hair cells from aminoglycoside-induced ototoxicity. **Journal of Neuroscience Research**, v. 79, n. 5, p. 644–651, 2005.

JOHNSON, J. D.; FLESHNER, M. Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72. **Journal of Leukocyte Biology**, v. 79, n. 3, p. 425–434, 30 dez. 2005.

KIM, M.; WISCHMEYER, P. E. Glutamine. **World Review of Nutrition and Dietetics**, v. 105, p. 90–96, 2013.

KIM, Y. H. et al. Geranylgeranylacetone ameliorates acute cochlear damage caused

by 3-nitropropionic acid. **NeuroToxicology**, v. 31, n. 3, p. 317–325, 2010.

KONINGS, A. et al. Variations in HSP70 genes associated with noise-induced hearing loss in two independent populations. **European journal of human genetics : EJHG**, v. 17, n. 3, p. 329–335, 2009.

KRAUSE, M. et al. The Chaperone Balance Hypothesis : The Importance of the Extracellular to Intracellular HSP70 Ratio to Inflammation-Driven Type 2 Diabetes , the Effect of Exercise , and the Implications for Clinical Management. v. 2015, 2015.

KRAUSE, M.; RODRIGUES-KRAUSE, J. D. C. Extracellular heat shock proteins (eHSP70) in exercise: Possible targets outside the immune system and their role for neurodegenerative disorders treatment. **Medical Hypotheses**, v. 76, n. 2, p. 286–290, 2011.

LAYMAN, W. S. et al. Histone deacetylase inhibition protects hearing against acute ototoxicity by activating the Nf-κB pathway. **Cell death discovery**, v. 1, n. 15012, jan. 2015.

LIBERMAN, M. C.; BEIL, D. G. Hair cell condition and auditory nerve response in normal and noise-damaged cochleas. **Acta oto-laryngologica**, v. 88, n. 3–4, p. 161–76, 1979.

LIM, H. H. et al. Detection of HSP 72 synthesis after acoustic overstimulation in rat cochlea. **Hearing research**, v. 69, p. 146–150, 1993.

LINDQUIST, S.; CRAIG, E. A. The Heat-Shock Proteins. **Annual Review of Genetics**, v. 22, n. 1, p. 631–677, dez. 1988.

LO, W. C. et al. Dose-dependent effects of d-methionine for rescuing noise-induced permanent threshold shift in guinea-pigs. **Neuroscience**, v. 254, p. 222–229, 2013.

LOOI, L. M. et al. Hearing loss: a global health issue. **The Lancet**, v. 385, n. 9972, p. 943–944, 2015.

MATHEW, A.; MORIMOTO, R. I. Role of the heat-shock response in the life and death of proteins. **Annals of the New York Academy of Sciences**, v. 851, p. 99–111, 1998.

MEIRA, T. C.; SANTANA, V. S.; FERRITE, S. Gender and other factors associated with the use of hearing protection devices at work. **Revista de saúde pública**, v. 49, p. 1–8, jan. 2015.

MIKURIYA, T. et al. Geranylgeranylacetone, a heat shock protein inducer, prevents acoustic injury in the guinea pig. **Brain Research**, v. 1065, n. 1–2, p. 107–114, 2005.

MIKURIYA, T. et al. Attenuation of progressive hearing loss in a model of age-related hearing loss by a heat shock protein inducer, geranylgeranylacetone. **Brain Research**, v. 1212, p. 9–17, 2008.

MYERS, M. W. et al. Expression of the Major Mammalian Stress Protein in the Rat Cochlea Following Transient Ischemia. **The Laryngoscope**, v. 102, n. 9, p. 981–987, set. 1992.

NEELY, J. G.; THOMPSON, A. M.; GOWER, D. J. Detection and localization of heat shock protein 70 in the normal guinea pig cochlea. **Hearing Research**, v. 52, n. 2, p. 403–406, 1991.

NELSON, D. I. et al. The global burden of occupational noise-induced hearing loss. **American journal of industrial medicine**, v. 48, n. 6, p. 446–458, 2005.

NEWSHOLME, P. et al. Glutamine and glutamate as vital metabolites. **Brazilian Journal of Medical and Biological Research**, v. 36, n. 2, p. 153–163, 2003.

NUTTALL, A. L. Sound-Induced Cochlear Ischemia/Hypoxia as a Mechanism of Hearing Loss. **Noise & health**, v. 2, n. 5, p. 17–32, jan. 1999.

OGUCHI, T. et al. Inner hair cells of mice express the glutamine transporter SAT1. **Hearing Research**, v. 292, n. 1–2, p. 59–63, 2012.

PETRY, É. R. et al. Alanyl-glutamine and glutamine plus alanine supplements improve skeletal redox status in trained rats: Involvement of heat shock protein pathways. **Life Sciences**, v. 94, n. 2, p. 130–136, 2014.

POIRRIER, A L. et al. Oxidative stress in the cochlea: an update. **Current medicinal chemistry**, v. 17, p. 3591–3604, 2010.

RITOSSA, F. A new puffing pattern induced by temperature shock and DNP in drosophila. **Experientia**, v. 18, n. 12, p. 571–573, 1 dez. 1962.

ROGERO, M. M. et al. Effect of alanyl-glutamine supplementation on plasma and tissue glutamine concentrations in rats submitted to exhaustive exercise. **Nutrition**, v. 22, n. 5, p. 564–571, 2006.

ROTH, E. Nonnutritive effects of glutamine. **The Journal of nutrition**, v. 138, n. 10, p. 2025S–2031S, 2008.

RYBAK, L. P. et al. Cisplatin ototoxicity and protection: clinical and experimental studies. **The Tohoku journal of experimental medicine**, v. 219, n. 3, p. 177–86, 2009.

SANO, H. et al. Effect of geranylgeranylacetone on gentamycin ototoxicity in rat cochlea culture. **Auris Nasus Larynx**, v. 34, n. 1, p. 1–4, 2007.

SINGLETON, K. D.; WISCHMEYER, P. E. Glutamine's protection against sepsis and lung injury is dependent on heat shock protein 70 expression. **American journal of physiology. Regulatory, integrative and comparative physiology**, v. 292, n. 5, p. R1839–R1845, 2007.

SLIWINSKA-KOWALSKA, M.; PAWELCZYK, M. Contribution of genetic factors to noise-induced hearing loss: a human studies review. **Mutation research**, v. 752, n. 1, p. 61–5, 2013.

SONE, M. et al. Upregulation of HSP by geranylgeranylacetone protects the cochlear lateral wall from endotoxin-induced inflammation. **Hearing Research**, v. 204, n. 1–2, p. 140–146, 2005.

SUGAHARA, K. et al. Heat shock transcription factor HSF1 is required for survival of sensory hair cells against acoustic overexposure. **Hearing Research**, v. 182, p. 88–96, 2003.

TAK, S.; DAVIS, R. R.; CALVERT, G. M. Exposure to hazardous workplace noise and use of hearing protection devices among US workers--NHANES, 1999-2004. **American journal of industrial medicine**, v. 52, n. 5, p. 358–371, 2009.

TALEB, M. et al. Hsp70 inhibits aminoglycoside-induced hearing loss and cochlear hair cell death. **Cell Stress and Chaperones**, v. 14, p. 427–437, 2009.

THOMPSON, A. M.; NEELY, J. G. Induction of heat shock protein in interdental cells by hyperthermia. **Otolaryngology-Head and Neck Surgery**, v. 107, n. 6_part_1, p. 769–774, dez. 1992.

WANG, Y.; LIBERMAN, M. C. Restraint stress and protection from acoustic injury in mice. **Hearing Research**, v. 165, n. 1–2, p. 96–102, 2002.

WORLD HEALTH ORGANIZATION. Community-Based Rehabilitation Promoting Ear and Hearing Care through CBR. p. 28, 2012.

XIAO, X. et al. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. **EMBO Journal**, v. 18, n. 21, p. 5943–5952, 1999.

YANG, M. et al. Association of hsp70 polymorphisms with risk of noise-induced hearing loss in Chinese automobile workers. **Cell stress & chaperones**, v. 11, n. 3, p. 233–239, 2006.

YOSHIDA, N.; KRISTIANSEN, A.; LIBERMAN, M. C. Heat stress and protection from permanent acoustic injury in mice. **The Journal of neuroscience**, v. 19, n. 22, p. 10116–10124, 1999.

YURINSKAYA, M. et al. The Fate of Exogenous Human HSP70 Introduced into Animal Cells by Different Means. 2015.

6 ANEXOS – NORMAS DAS REVISTAS

6.1 HEARING RESEARCH

INTRODUCTION
The aim of the journal is to provide a forum for papers concerned with basic auditory mechanisms. Emphasis is on experimental studies, but theoretical papers will also be considered. The journal publishes original research papers, review and mini- review articles, rapid communications, method/protocol and perspective articles. Papers submitted should deal with auditory neurophysiology, ultrastructure, psychoacoustics and behavioural studies of hearing in animals, and models of auditory functions. Papers on comparative aspects of hearing in animals and man, and on effects of drugs and environmental contaminants on hearing function will also be considered. Clinical papers will not be accepted unless they contribute to the understanding of normal hearing functions. Authors may suggest one or two reviewers from the Editorial Board for consideration by the Editor.

Please note that authors must now include bulleted " Research highlights" and may also include a " Graphical abstract" with their article (see below).

Types of papers
Research Papers
These articles should deal with original research not previously published or being considered for publication elsewhere. These papers should provide a survey, evaluation and critical interpretation of recent research results and concepts in the fields covered by the Journal.

Review Articles
These are exhaustive reviews on a specific topic of hearing research. Authors should always endeavor to make their reviews understandable to a broad range of auditory scientists. Review submissions are typically 10,000 words.

Short review
These are shorter reviews intended either to draw attention to developments in a specific or emerging field. Short review submissions are typically 3,000 words.

Short communication
The purpose of this new category is to provide an extremely rapid decision (within 7 days) on papers of unusual importance and report definitive observations. Text limit: 2,500 words and a maximum of 4 figures and/or tables. Within seven days of submission, authors will receive a "yes/no" decision. If the editors and reviewers deem a "yes" decision then the revision must consist only of minor edits and the revised manuscript must be returned in five days.

Technical note
These articles will describe techniques or protocols of particularly broad interest or especially timely for the auditory audience. The manuscripts should be written as concisely as possible but should contain all necessary details to allow replication of the results. These manuscripts should be subdivided by short bold headings referring to Introduction, Rationale, Description of the methods, Results and Conclusions.

Opinion paper
This is by invitation and is a composite article is intended to give the experts in a given field an opportunity to voice their personal opinion. Each expert has given their own personal view on the topic and at the end of their commentary they have suggested several experiments that would be required for the decisive. These experiments are presently lacking but if successfully performed would have an enormous impact on our understanding of the chosen topic. The journal welcomes proposals for topics that fall within the scope of the journal and proposals should be sent to the Editor-In-Chief for evaluation.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
• E-mail address
• Full postal address

All necessary files have been uploaded:
Manuscript:
• Include keywords
• All figures (include relevant captions)
• All tables (including titles, description, footnotes)
• Ensure all figure and table citations in the text match the files provided
• Indicate clearly if color should be used for any figures in print
Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

Further considerations
• Manuscript has been 'spell checked' and 'grammar checked'
• All references mentioned in the Reference List are cited in the text, and vice versa
• Permission has been obtained for use of copyrighted material from other sources (including the Internet)
• Relevant declarations of interest have been made
• Journal policies detailed in this guide have been reviewed
• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

Before You Begin

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Human and animal rights

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed.

Declaration of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial,

personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. More information.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see 'Multiple, redundant or concurrent publication' section of our ethics policy for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck.

Contributors
Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts Before the accepted manuscript is published in an online issue Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include:

- The reason the name should be added or removed or the author names rearranged.
- Written confirmation (email, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that:

- Journal Managers will inform the Journal Editors of any such requests.
- Publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue
Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before**submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of open access articles is determined by the author's choice of user license.

Author rights
As an author you (or your employer or institution) have certain rights to reuse your work. More information.

Elsevier supports responsible sharing
Find out how you can share your research published in Elsevier journals.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies
Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the Open Access Publication Fee. Details of existing agreements are available online.

Open access

This journal offers authors a choice in publishing their research:

Open access
• Articles are freely available to both subscribers and the wider public with permitted reuse.
• An open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.
Subscription
• Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.
• No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)
Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as

they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)
For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is **USD 2150**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access
Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form. Find out more.

This journal has an embargo period of 12 months.

Elsevier Publishing Campus
The Elsevier Publishing Campus (www.publishingcampus.com) is an online platform offering free lectures, interactive training and professional advice to support you in publishing your research. The College of Skills training offers modules on how to prepare, write and structure your article and explains how editors will look at your paper when it is submitted for publication. Use these resources, and more, to ensure that your submission will be the best that you can make it.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Sumission address
To submit your paper use the online submission page of this journal at https://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=HEARES

Preparation

Use of wordprocessing software
It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. Do not embed "graphically designed" equations or tables, but prepare these using the wordprocessor's facility. When preparing tables, if you are using a table grid, use only one grid for each

individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). Do not import the figures into the text file but, instead, indicate their approximate locations directly in the electronic text and on the manuscript. See also the section on Electronic illustrations.
To avoid unnecessary errors you are strongly advised to use the "spell-check" and "grammar-check" functions of your wordprocessor. Reviewer line numbers must be inserted in the left-hand margin.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Experimental
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail

address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract
Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images and in accordance with all technical requirements: Illustration Service.

Highlights
Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Math formulae
Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Artwork

Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Colour in print is free of charge for Methodology papers and for invited reviews.

Cover illustration: Authors are encouraged to submit interesting figures for possible publication on the front cover of an issue of this journal; the figure should be part of or related to thier article.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links
Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

A DOI can be used to cite and link to electronic articles where an article is in-press and full citation details are not yet known, but the article is available online. A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884. Please note the format of such citations should be in the same style as all other references in the paper.

Web references
As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.),

should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:
http://open.mendeley.com/use-citation-style/hearing-research
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference Style
Reference Style

Text: Indicate references within the text in parentheses by author name, then year.
For example: (Smith, et al., 2007)

List: References should be listed in alphabetical order by author's last name.
For example:

Bibel,M.,Hoppe,E.,Barde,Y.A.,1999.Biochemical and functional interactions between the neurotrophin receptors trk and p75(NTR).EMBO Journa18, 616-622.

Brors, D.,Hansen,S.,Mlynski,R.,Volkenstein,S.,Aletsee,C.,Sendtner,M.,Ryan,A.F., Dazert, S.,2008.Spiral ganglion outgrowth and hearing development in p75 (NTR)-deficient mice. Audiology and Neuro-otology 13, 388-395.

Chu, G.K.T.,Yu,W.,Fehlings,M.G.,2007. The p75 neurotrophin receptor is essential for neuronal cell survival and improvement of functional recovery after spinal cord injury. Neuroscience 148, 668-682.

Davis,R.R.,Newlander,J.K.,Ling,X.B.,Cortopassi,G.A.,Krieg,E.F.,Erway,L.C.,2001. Genetic basis for susceptibility to noise-induced hearing loss in mice. Hearing Research155, 82-90.

Dechant, G.,Barde,Y.A.,2002.The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. Nature Neuroscience 5, 1131-1136.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations.

Video

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Data linking
If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that give them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

ARTICLE ENRICHMENTS

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available.

Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Interactive MATLAB Figure Viewer

This journal features the Interactive MATLAB Figure Viewer, allowing you to display figures created in MATLAB in the .FIG format in an interactive viewer next to the article. More information and submission instructions.

3D neuroimaging

You can enrich your online articles by providing 3D neuroimaging data in NIfTI format. This will be visualized for readers using the interactive viewer embedded within your article, and will enable them to: browse through available neuroimaging datasets; zoom, rotate and pan the 3D brain reconstruction; cut through the volume; change opacity and color mapping; switch between 3D and 2D projected views; and download the data. The viewer supports both single (.nii) and dual (.hdr and .img) NIfTI file formats. Recommended size of a single uncompressed dataset is maximum 150 MB. Multiple datasets can be submitted. Each dataset will have to be zipped and uploaded to the online submission system via the '3D neuroimaging data' submission category. Please provide a short informative description for each dataset by filling in the 'Description' field when uploading a dataset. Note: all datasets will be available for downloading from the online article on ScienceDirect. If you have concerns about your data being downloadable, please provide a video instead. More information.

3D radiological data

You can enrich your online article by providing 3D radiological data in DICOM format. Radiological data will be visualized for readers using the interactive viewer embedded within your article, and will enable them to: browse through available radiological datasets; explore radiological data as 2D series, 2D orthogonal MPR, 3D volume rendering and 3D MIP; zoom, rotate and pan 3D reconstructions; cut through the volume; change opacity and threshold level; and download the data. Multiple datasets can be submitted. Each dataset will have to be zipped and uploaded to the online submission system via the '3D radiological data' submission category. The recommended size of a single uncompressed dataset is 200 MB or less. Please provide a short informative description for each dataset by filling in the 'Description' field when uploading each ZIP file. Note: all datasets will be available for download from the online article on ScienceDirect. So please ensure that all DICOM files are **anonymized** prior to submission. More information.

Interactive plots

This journal enables you to show an Interactive Plot with your article by simply submitting a data file. Full instructions.

After Acceptance

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us

in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

6.2 PLOS ONE

Style and Format

File format	Manuscript files can be in the following formats: DOC, DOCX, RTF, or PDF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
Length	Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.
Font	Use a standard font size and any standard font, except for Symbol font.
Headings	Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.
Layout	Manuscript text should be double-spaced. Do not format text in multiple columns.
Page and line numbers	Include page numbers and line numbers in the manuscript file.
Footnotes	Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.
Language	Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.
Abbreviations	Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.
Reference style	PLOS uses "Vancouver" style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.

Equations	We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor is acceptable. Avoid using MathType or Equation Editor to insert single variables (e.g., "$a^2 + b^2 = c^2$"), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values. Do not use MathType or Equation Editor for only a portion of an equation. Rather, ensure that the entire equation is included. Avoid "hybrid" inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.
Nomenclature	Use correct and established nomenclature wherever possible.

Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.
Drugs	Provide the Recommended International Non-Proprietary Name (rINN).
Species names	Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).
Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).

Copyediting manuscripts

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service."

Submissions are not copyedited before publication.

Submissions that do not meet the *PLOS ONE* publication criterion for language standards may be rejected.

Manuscript Organization

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section	*The following elements are required, in order:* • Title page: List title, authors, and affiliations as first page of manuscript • Abstract • Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* • Materials and Methods • Results • Discussion • Conclusions (optional)
Ending section	*The following elements are required, in order:* • Acknowledgments • References • Supporting information captions (if applicable)
Other elements	• Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. • Tables are inserted immediately after the first paragraph in which they are cited. • Supporting information files are uploaded separately.