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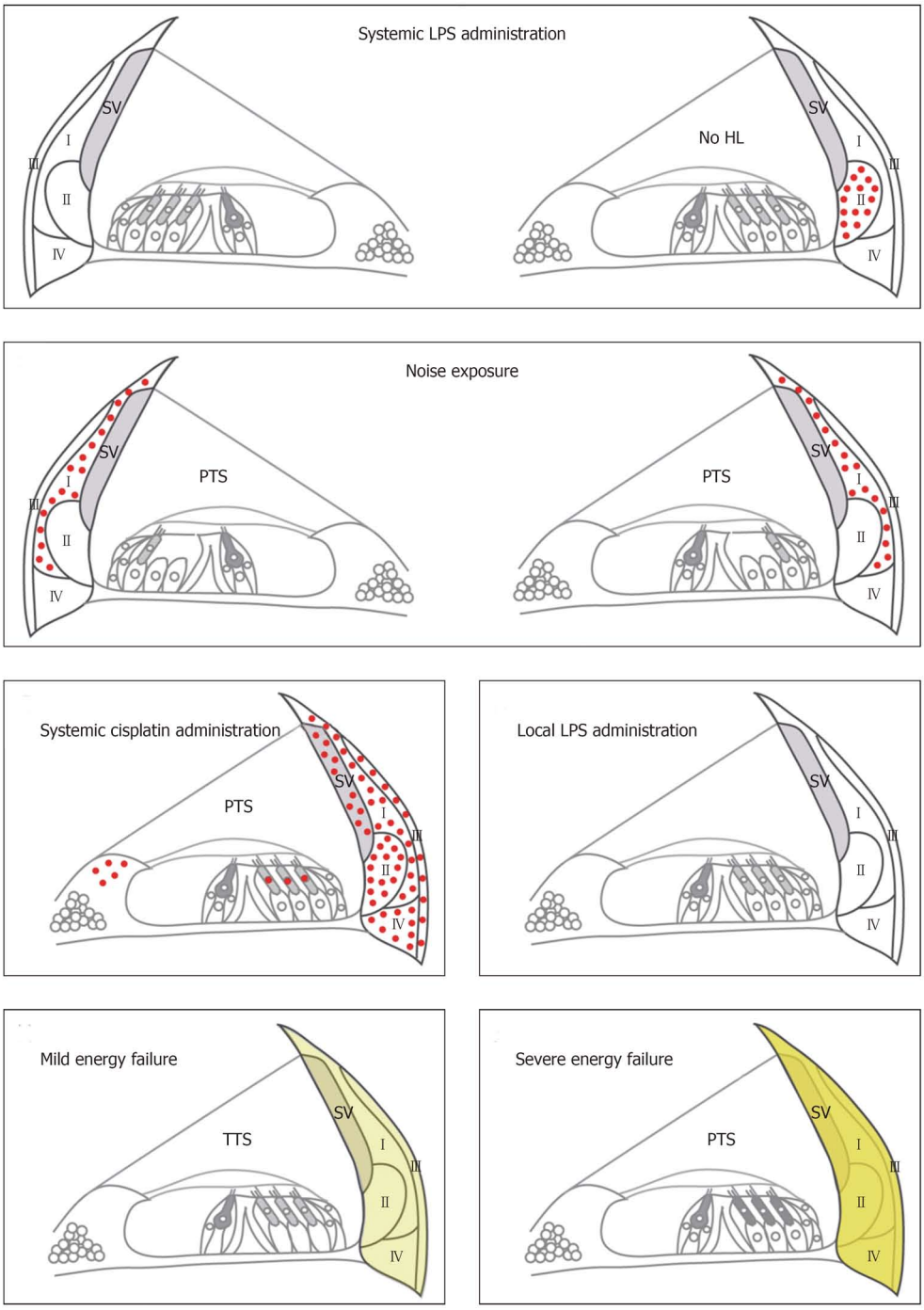

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FRONTIER

Cause of idiopathic sudden sensorineural hearing loss: The stress response theory

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Abstract

The stress response theory is a relatively new concept about the cause of idiopathic sudden sensorineural hearing loss (ISHL). A number of possible etiologies have been proposed in the literature, as discussed in this paper, but each proposed etiology has been both supported and refuted in the literature. However, the stress response theory can integrate hypotheses that have been advocated so far. The word "stress" refers to a constellation of physical and psychological stimuli including systemic viral and bacterial illness, systemic inflammatory disorders, and physical, mental or metabolic stress. Numerous studies have demonstrated adverse effects of systemic stress on health. Stress causes changes in the immune system and cytokine network through activation of the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system. Several types of catecholamine and cytokine receptors are in the cochlea cells other than capillary cells, and then they can respond to systemic stressors. However, there are few studies examining how systemic stress is associated with cochlear dysfunction. The stress response theory addresses this question. In the theory, a variety of stressors and risk factors contribute to the onset of ISHL in varying degrees. The lateral wall of the cochlea has very unique responses to systemic stressors. It plays a critical role in causing ISHL. Systemic stressors converge at the lateral wall and trigger pathological activation of nuclear factor κ-light-chain-enhancer of activated B cells, a transcriptional factor known as a stress sensor. This activation enhances local expression of genes associated with immune and inflammatory system, resulting in cochlear dysfunction. We review the original stress response theory advocated by Adams *et al* and the integrative stress response theory that integrates our knowledge about the etiologies of ISHL so far.

Key words: Cause; Idiopathic sudden sensorineural hearing loss; Lateral wall; Nuclear factor κ-light-chain-enhancer of activated B cells; Stress

Core tip: The present review focuses on the following four points. First, it summarizes etiologies proposed in the last decade to confirm what we know about the cause of idiopathic sudden sensorineural hearing loss (ISHL). Second, it reviews how systemic stressors affect the human body and the cochlea. Third, it reviews the characteristics of the lateral wall that show unique responses to systemic stressors. Finally, it reviews a relatively new concept about the cause of ISHL, the stress response theory, which integrates our knowledge of the cause of ISHL.

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INTRODUCTION

Idiopathic sudden sensorineural hearing loss (ISHL) is a moderately common otologic disorder characterized by new onset of unilateral reversible or irreversible sensorineural hearing loss, which generally develops over minutes or several hours. However, the etiology remains unknown. During the past decade, a number of papers on proposed etiologies have been published, including those on vascular disturbance, viral infection, and immune-mediated mechanisms. Table 1 summarizes papers about the cause of ISHL published during this decade[1-73]. Papers for and against vascular etiologies with analysis of genetic polymorphism are increasing. Yet, there is no conclusive evidence, and many different treatments exist for ISHL[74]. In fact, Nosrati-Zarenoe *et al*[75] reported no significant difference in outcomes between treated and non-treated patients (300 patients in total).

To develop and apply the best treatment for ISHL, we must reveal the pathophysiology. Most papers focus on one cause of the disease, and each proposed etiology has papers that support and refute it, as shown in Table 1. On the other hand, the stress response theory can integrate the various hypotheses proposed up to this point, and can explain the clinical characteristics of ISHL. Originally, the theory was advocated by Merchant *et al*[53] and Adams[76]. They proposed that ISHL might be a result of pathologic activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) in the cochlear lateral wall. NF-κB is activated by various stressors, acting as a "stress sensor". It plays a pivotal role in regulating expression of genes associated with immune system and inflammatory responses. For example, interleukin (IL)-6, tumor necrosis factor-α (TNF-α), inducible nitric oxide (iNOS), and intercellular adhesion molecule 1 (ICAM-1) are NF-κB responsive genes[77]. The authors demonstrated that NF-κB was activated in the unilateral cochlear lateral wall by the systemic stressor, *i.e.*, intraperitoneal bacterial endotoxin lipopolysaccharide (LPS) injection, but not by the local stressor, *i.e.*, intratympanic LPS injection (Figure 1A and D). Then, they speculated that ISHL would be the result of pathologic NF-κB activation responding to the systemic stressor.

In the present review, we describe the association of stress and the onset of ISHL, extending the original concept of the stress response theory. To begin, we will quickly review the influence of the chronic psychosocial and physiological stressors on the human body.

OVERVIEW OF CHRONIC STRESS EFFECTS ON HUMAN BODY

Selye *et al*[78,79] defined stress response as the body's nonspecific response when a human being is subjected to stressors, including psychosocial, physical, and biological stimuli. More than half a century ago, Selye *et al*[80] showed that stress caused damage to organs like the heart and the kidney. In recent years, there is accumulating evidence that chronic stress results in many diseases including dermatitis, depression, cardiovascular disease, osteopenia/osteoporosis, immune suppression, and insulin resistance through the activation of the hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS)[81-83].

Briefly, stressors induce release of corticotropin-releasing hormone (CRH) from the hypothalamus, CRH induces adrenocorticotropic hormone (ACTH) release from the anterior pituitary, ACTH induces glucocorticoid (GC) secretion from the adrenal cortex, and GC induces norepinephrine (NEP) and epinephrine (EP) release from the adrenal medulla. Stressors and CRH also activate the locus coeruleus of the brainstem, eliciting an SNS response and resulting in NEP and EP release. Furthermore, chronic psychological stress results in a decrease in the sensitivity of immune cells to GC that normally terminates the inflammatory response, which then increases a variety of disease risks[84].

Possible direct effects of the autonomic nervous system on the cochlea

It is well known that the cardiovascular system is directly regulated by the NEP and EP from the SNS, and acetylcholine from the parasympathetic nervous system. In addition, converging evidence from animal and human studies indicates that there is an association between stress and cardiovascular disease[81]. However, the effect of these systemic stress-induced catecholamines on the cochlea remains unknown.

Several types of adrenergic and muscarinic acetylcholine receptors are located in the cochlea and in the endolymphatic sac, as well as in blood vessels like the spiral modiolar artery (Table 2)[85-94]. Their exact functions and synapse formation with autonomic nerve fibers are not clear. However, the stress-induced circulating EP and NEP increase will relay the SNS activity to the inner ear. Parasympathetic nervous system activity will also affect the inner ear, because the activity can be relayed to the whole body by the circulating acetylcholine-synthesizing T cells[95], even if the parasympathetic nerve and the cochlea cells do not have synaptic formation.

Considering that these receptors are on the vessels of the cochlea, the lateral wall, and the endolymphatic sac, they probably contribute to the following functions: (1) regulation of the blood flow; (2) maintenance of the inner ear lymphatic ion homeostasis; and (3) enhancement of the stress response in the lateral wall. We will describe these again later in this review.

Stress-induced dysfunction of immune system through the decrease of natural killer cell activity

Chronic stress disrupts immune system homeostasis and induces susceptibility to infectious and autoimmune diseases[96,97]. Furthermore, there are frequent associations between infectious diseases and autoimmune diseases[98]. The natural killer (NK) cell has critical roles in resistance against both viral and bacterial infections[99,100], and in

Table 1 Proposed causes of idiopathic sudden sensorineural hearing loss during this decade

Main category	Subcategory	Significantly associated factors	NOT significantly associated factor	Ref.
Vascular impairment		MTHFR poly., homocysteine	FV poly., PT poly., AT, LAC, protein S, protein C	[1,2]
	Medical history	MTHFR poly., FV Leiden poly., folate, cardioV risk factors		[3]
	Medical history	Platelet GlyIa poly.	Platelet GlyIIIa poly., Framingham cardioV risk factors, FV Leiden poly., PT poly., history of cardioV events, brain stroke, antiphospholipid syndrome	[4,5]
		PAI-1 poly.		[6]
	Medical history	CFH poly. with DM	HT, lipid	[7]
	Medical history	Low FMD of the brachial artery	Low C-IMTI, LDL, cardioV risk factors	[8]
		Vertebrobasilar junction angulation		[9]
		High global oxidative stress index		[10]
			FV Leiden poly., PT poly.	[11]
	Medical history	SBP, personal/family history cardioV events	FV poly., PT poly., HT, DM, lipid, smoking, personal/family history or in the presence of thrombotic factors	[12]
		PKCH poly., MTHFR poly.		[13,14]
		Cho, LDL		[15]
		Cho, LDL, unsaturated fatty acid, coenzyme Q10, folate, homocysteine	MTHFR poly., FV poly., PT poly., antithrombin Ⅲ, protein C and S, D-dimer, FG, activated protein C resistance	[16-18]
		MTHFR poly., FV Leiden poly., PT poly., platelet GlyIIIaA1/A2 poly., homocysteine, Cho, FG, folate		[19,20]
		MTHFR poly.	FV, PT, EPCR, PAI-1	[21]
	Medical history	ICAM-1, VCAM-1	Cho, triglyceride, FG, ESR, smoking, DM	[22]
		MTHFR poly. with MTR poly., MTR poly.	MTHFR poly. alone	[23]
		FV Leiden poly.	PT poly.	[24]
	Auto-immunity	Cho, homocysteine, PAI-1, anticardiolipin antibodies	FV Leiden poly., FII poly., antithrombin, protein C and S, lupus anticoagulant, lipoprotein(a)	[25]
		eNOS poly.		[26]
		FMD		[27]
		Whole blood viscosity, erythrocyte deformability index, activated clotting time, clot rate, PAI-1 antigen, factor Ⅷ:C	Plasma viscosity, FG	[28]
	Auto-immunity	Antiendothelial cell antibody		[29,30]
Cytokine		IL-1B poly., TNF-β poly.		[31,32]
		TNF-α	IL-10, IL-12	[33]
		IL-6 poly.	IL-4R poly., IL-10 poly., TNF-α poly., TNFRSF1B poly., VEGF poly.	[34]
		IL-1A poly.	IL-1B poly.	[35]
	Vascular impairment		IL-6, IL-8, ICAM-1, VCAM-1, E-selectin, MCP-1, lipid, FG	[36]
		TNF-α, sCD40, sCD40L, T lymphocyte, CD40, cyclooxygenase 2, CD38 positive T or B lymphocyte	Monocyte, macrophage	[37]
Cellular stress		HSP70 poly.		[38]
			GPX1 poli., PON1 poli., PON2 poli., SOD2 poli.	[39]
			GST poly., CYP poly.	[40]
	Auto-immunity		Anti-HSP70 antibody, TNF-α, ESR, ANA, antiphospholipid antibody	[41]
	Auto-immunity	Anti-HSP70 antibody, anti-phospholipids antibody		[42]
		HSP70		[43]
			GST poly.	[44]
Infection		IgA to HSV1	IgG and IgM to CMV, VZV, HSV1, and HSV2. IgA to CMV, VZV, and HSV2	[45]
			Borrelia	[46]
			Herpes zoster	[47]
		Recent subclinical viral infection (cytomegalovirus, herpes simplex, Epstein-Barr virus), toxoplasmosis infections		[48]
			Enterovirus, cytomegalovirus, Epstein-Barr virus	[49]
Auto-immunity			Anti-double stranded DNA, RF, antiphospholipid IgG and M, antinuclear antibody, complements C3 and C4	[50]
		T cell responding to cochlin		[51]
Stress response theory		Neutrophil, NKCA, IL-6	TNF, hCRP	[52]
		Histological evidence of severe osmotic stress of the organ of Corti		[53]

Medical history		HIV		[54]
	Vascular impairment	SLE		[55]
	Vascular impairment	AMI		[56]
		Migrane with HT		[57]
	Vascular impairment	ED		[58]
	Vascular impairment	DM		[59]
		Chronic kidney disease with and without DM		[60]
		Allergy		[61]
		Male with OSA	Female with OSA	[62]
	Vascular impairment	CardioV risk factors, DM, Cho		[63]
		Family history of ISHL		[64]
	Vascular impairment	CerebroV stroke		[65]
Other aetiologies			Aquaporin 4 and 5 poly., estrogen receptor α poly.	[66]
		Round window membrane rupture		[67]
		Endolymphatic hydrops		[68]
			Eustachian tube dysfunction	[69]
		General anaesthesia		[70]
			Month, weather	[71]
		HLA-DQB1 and -DRB1		[72]
			Season, weather	[73]

Papers on human studies are categorized by the proposed etiologies. Papers that deduce possible etiologies from the effect of a treatment are excluded. For example, Kang *et al*[174] stated that the cause might be reactive oxygen metabolites produced by inner ear ischemia or inflammation, because high dose vitamin C was effective. Such papers are excluded from this table in order to save space, although they are noteworthy. AMI: Acute myocardial infarction; AT: Antithrombin; C-IMT: Carotid intima-media thickness; CardioV: Cardio vascular; CDL: CD ligand; CerebroV: Cerebrovascular; CFH: Complement factor H; Cho: Cholesterol; CMV: Cytomegalovirus; CYP: Cytochrome P450; DM: Diabetes mellitus; E-selectin: Endothelial selectin; ED: Erectile dysfunction; eNOS: Endothelial nitric oxide synthase; EPCR: Endothelial cell protein C receptor; ESR: Erythrocyte sedimentation rate; FG: Fibrinogen; FMD: Flow-mediated dilatation; FII: Factor II; FV: Factor V; Gly: Glycoprotein; GPX: Glutathione peroxidase; GST: Glutathione S-transferases; hCRP: High sensitivity C-reactive protein; HIV: Human immunodeficiency virus; HLA: Human leukocyte antigen; HSP: Heat shock protein; HSV: Herpes simplex virus; HT: Hypertension; ICAM-1: Intercellular adhesion molecule 1; IL: Interleukin; ISHL: Idiopathic sudden sensorineural hearing loss; LAC: Lupus anticoagulant; LDL: Low density lipoprotein; MCP-1: Monocyte chemoattractant protein 1; MTHFR: Methylene tetrahydrofolate reductase; MTR: Methionine synthase; NKCA: Natural killer cell activity; OSA: Obstructive sleep apnea; PAI-1: Plasminogen activator inhibitor-1; PKCH: Protein kinase C-Eta; poly.: Polymorphism; PON: Paraoxonase; PT: Prothrombin; RF: Rheumatoid factor; SBP: Systolic blood pressure; SLE: Systemic lupus erythematosus; sCD: Soluble cluster of differentiation; SOD: Superoxide dismutase; TNF: Tumor necrosis factor; TNFRSF1B: Tumor necrosis factor receptor superfamily 1b; VCAM-1: Vascular cell adhesion molecule 1; VEGF: Vascular endothelial growth factor; VZV: Varicella-zoster virus.

Table 2 Receptors of catecholamine in the cochlea

Location	Adrenergic receptor					Cholinergic receptor			
	α1	α2	β1	β2	[1]β	M1	M2	M3	M5
IHC	○		○			○		○	○
OHC	○		○			○		○	
Deiters' cells			○	○		○	○	○	○
Hensen's cells			○	○					
Outer sulcus			○						
Stria vascularis[2]				○					
Strial marginal cell			○						○
Capillaries in the stria vascularis								○	
Spiral ligament[2]			○	○		○	○	○	○
Rissener's membrane					○				
Spiral ganglion	○		○	○		○		○	○
Nerve fibers approaching HCs	○		○	○					
Efferent fibers of the intraganglionic spiral bundle							○		○
Spiral modiolar artery	○	○							
Endolymphatic sac				○					

[1]The specific receptor subtype, β1 or β2, was not determined; [2]Note that the receptors were detected in tissues apart from blood vessels. IHC: Inner hair cell; OHC: Outer hair cell.

regulating autoimmunity[101]. However, NK cell activity (NKCA) is reduced by chronic stress like fatigue, stressful life events, inability to cope with stress, and shortness of sleep[102-106]. Therefore, such chronic stress has the potential to put the host in danger of infectious and autoimmune disease.

Association of stress and cytokines

IL-1, IL-6, and TNF are well known pro-inflammatory cytokines. They are pleiotropic and work as both effectors and regulators of stress response composed of the HPA axis and the SNS[107-109]. Nitric oxide (NO) is also involved in the HPA axix response[110].

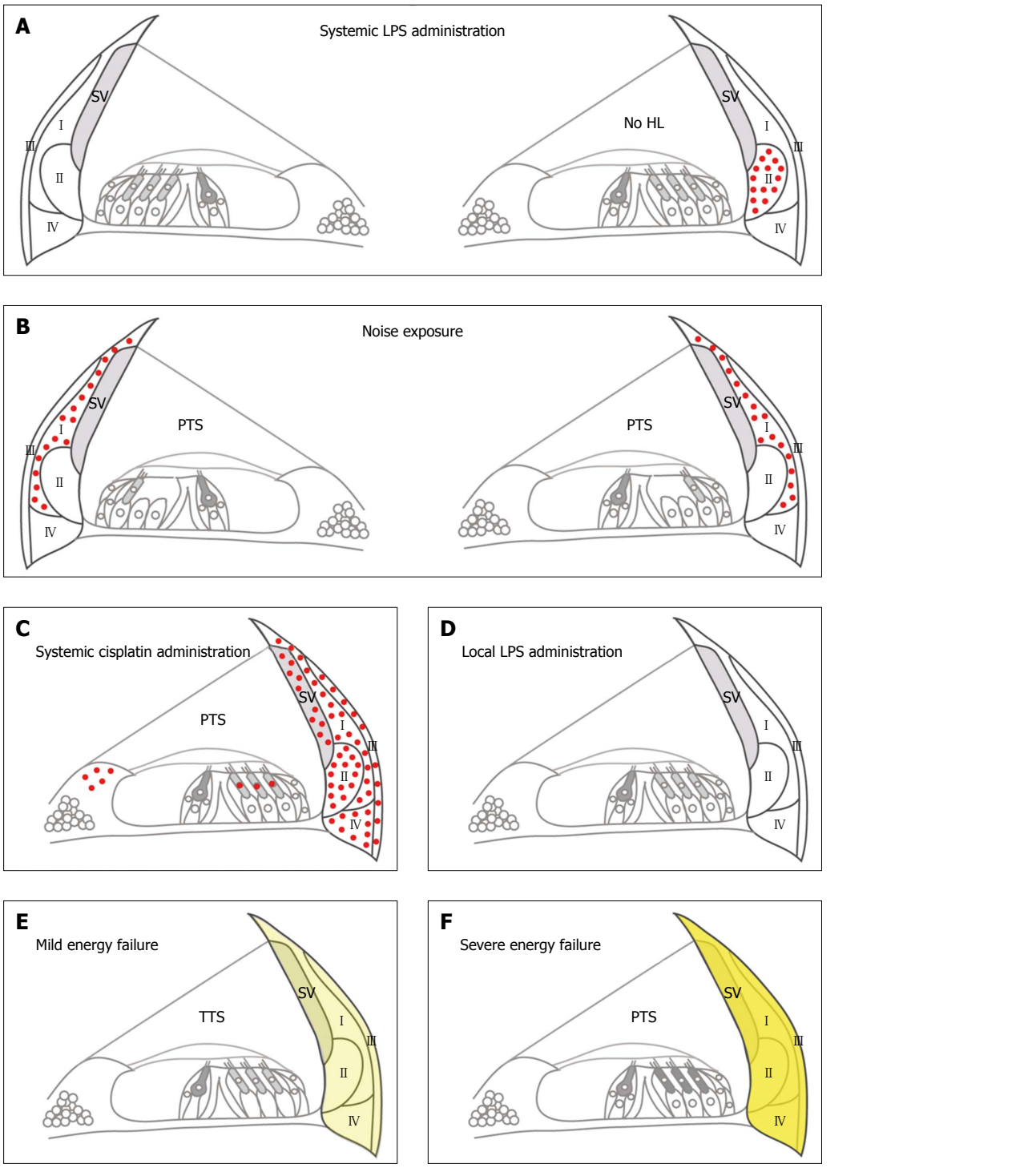

Figure 1 Characteristics of the lateral wall stress response. A-D: The lateral wall is composed of the stria vascularis (SV) and the spiral ligament, in which there are four types of fibrocytes (Ⅰ-Ⅳ). Intraperitoneal lipopolysaccharide (LPS) injection mainly activates nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (red dots) in the type Ⅱ fibrocytes asymmetrically between the two ears. However, local LPS injection (*i.e.*, intratympanic injection) does not induce the activation. Noise exposure activates NF-κB in type Ⅰ fibrocytes symmetrically at first. Systemic cisplatin injection activates NF-κB in the whole lateral wall, outer hair cells (HCs), and the spiral limbus, and causes HC loss; E: Mild energy failure of the lateral wall causes transient threshold shift (TTS); F: However, severe energy failure causes permanent threshold shift (PTS) with degeneration of cochlear lateral wall, and mild degeneration of the organ of Corti. HL: Hearing loss.

IL-1 is a potent pro-inflammatory cytokine[111], and is produced centrally and periphery following exposure to immunological and psychological stressors[108]. It directly activates the HPA axis and central nervous system, and can even cause depressive symptoms. IL-1 is also known to induce IL-6 strongly[107].

IL-6 is induced by stress as well as by IL-1. Stress-induced increases in IL-6 are a robust finding, and increases are typically higher in adverse psychological conditions[107]. Work stress is associated with an enhancement of IL-6 production by leukocytes before and after infectious stressor and with a lower capacity of GC to suppress IL-6 production[112].

Local and circulating IL-6 can influence the whole body *via* classical- and trans-signaling, respectively. In classical signaling, IL-6 works in cells that express the membrane-bound IL-6 receptor, but only few cells express it, so this signaling works locally. In trans-signaling, a complex of circulating IL-6 bound to the IL-6 receptor, which occurs naturally or by cleavage from apoptotic neutrophil, can control inflammatory response through binding with glycoprotein (gp130), displayed by all cells[113].

The underlying mechanism of IL-6 increase under stress is associated with activation of NF-κB[114,115]. Cortisol and catecholamines in the HPA axis and the SNS induce and enhance NF-κB activation under psychosocial stress[114,116]. Then, NF-κB induces IL-6 expression. IL-6 is not only a transcriptional target of NF-κB, but also an activator of NF-κB[117,118]. Therefore, a positive feedback loop can be made between the two.

Chronic stress also disturbs the circadian rhythm of serum IL-6 levels. Although serum levels of IL-6 reflect circadian cycle, IL-6 decreases less during the night in individuals experiencing negative mood or fatigue than those experiencing uplift events[119]. Conversely, IL-6 elevation per se generates fatigue, fever, and sleep-related symptoms such as daytime somnolence[107].

Additionally, TNF-α also increases under chronic psychosocial stress[106,120-123]. There is in fact a personality type associated with high TNF-α, distressed personality[124]. It may be a possible reason why final health status is different among individuals under the same stress.

NO is generally identified as a molecule involved in neurotransmission, neuromodulation, controlling arterial diameter, and protecting blood vessels from deleterious consequences of platelet aggregation and activation of inflammatory responses[125,126]. It is also involved in the LPS-induced HPA axis response under basal conditions and during its adaptation to chronic social stress circumstances[110]. Excess NO induced by increased iNOS expression leads to the formation of a powerful oxidant, peroxynitrite. It results in cell death by many mechanisms, including lipid peroxidation, protein nitration, DNA damage, or the irreversible inhibition of respiration[127].

The description above concerns the stress response of the whole body. Next, we will review the characteristics of the lateral wall that play a leading role in the stress response theory.

CHARACTERISTICS OF THE COCHLEAR LATERAL WALL

An essential role for cochlear homeostasis

The lateral wall consists of the stria vascularis and the spiral ligament, in which there are four types of fibrocytes (Figure 1). The fibrocytes are classified based on general location, and localization of sodium-potassium-adenosine- triphosphatase (Na^+/K^+-ATPase) and the gap junction protein connexin 26[128-131]. They have a critical role in the potassium ion recycling mechanism[132], and could also be implicated in the mechanisms of glucose transport in the cochlea[133]. Type Ⅲ fibrocytes have even contractility and regulate tension of the basilar membrane, thereby determining auditory sensitivity[134].

In the stria vascularis, there are three types of cells that express multiple ion-transport apparatuses[135]. Therefore, the lateral wall is essential for maintaining cochlear homeostasis, and thus for normal hearing[128,135]. Degeneration of the lateral wall may be implicated in the survival of sensory cells[136].

Association between the extent of lateral wall dysfunction and the degree of hearing loss

Hoya *et al*[137], Mizutari *et al*[138] and Okamoto *et al*[139] demonstrated that different degrees of acute energy failure in the cochlear lateral wall cause different degrees of degeneration of the lateral wall fibrocytes, resulting in different degrees of hearing loss (Figure 1E and F). They administered the mitochondrial toxin 3-nitropropionic acid (3-NP) in the rat cochlea through the round window. Five hundred mmol/L 3-NP caused a permanent threshold shift of more than 80 dB at 8-20 kHz 3 h after 3-NP administration[139]. Marked degeneration of type Ⅱ fibrocytes, type Ⅳ fibrocytes, and cells in the stria vascularis were detected at the same time. Lateral wall degeneration was progressive for at least 14 d. In the organ of Corti, mitochondrial translocation in outer hair cells and mild degeneration of Deiters cells were observed 7 and 14 d after the administration, respectively.

On the other hand, 300 mmol/L 3-NP caused a completely reversible threshold shift at 8 kHz and degeneration of the lateral wall was not observed 3 wk after the 300 mmol/L 3-NP administration[138]. These suggest that mild energy failure of the lateral wall causes temporal and mild disturbance of cochlear homeostasis and temporal hearing loss without loss of inner ear cells. However, severe energy failure causes loss of the lateral wall cells, and then induces structural changes in the organ of Corti resulting in permanent hearing loss.

Wang *et al*[140] demonstrated that different intensities of an octave band noise (8-16 kHz) resulted in degeneration of different kinds of fibrocytes in the lateral wall and different degrees of hearing loss. In the lateral wall, 94 dB SPL noise caused loss of type Ⅳ fibrocytes alone, and resulted in only about 10 dB threshold shift at most. However, more than 112 dB SPL noise caused loss of type Ⅰ, Ⅱ, and Ⅳ fibrocytes, and resulted in more than 60 dB threshold shift.

These findings suggest that degree of the energy failure and the extent of the dysfunctional region in the lateral wall are the critical indicators of the degree of acute hearing loss.

Change of a region where NF-κB is activated in the lateral wall

NF-κB is activated in the lateral wall 24 h or earlier after loading stressors (Table 3)[141-146]. Interestingly, the region where NF-κB is activated changes in the lateral wall depending on the kind of stressor, the degree of the stress

Table 3 Nuclear factor κ-light-chain-enhancer of activated B cells activation and associated cytokine expression in the lateral wall

Animal	Stressor	Time point	NF-κB		Other factors			Ref.
			Response	Location	Factor	Response	Location	
CBA/CaJ mice	LPS, *ip*	24 h	Activation	Unilateral, Ⅱ >> Ⅰ, Lim.				[141]
	LPS, *ip* + dexamethasone, *ip*		No activation					
	Anti-CD3, *ip*		Activation	Ⅰ				
	Taxol, *ip*		Activation	Ⅰ				
	100 dB SPL		Activation	Bilateral, Ⅰ >> Ⅱ, Lim.				
CBA mice	117 dB SPL	4 h	Transcription	LW				[142]
		2-12 h[1] (4 h)			iNOS	Transcription	LW	
		0-12 h (6 h)			ICAM-1	Transcription	LW	
		2-12 h (6 h)			VCAM-1	Transcription	LW	
		14 h			ICAM-1	Expression	SV	
		14 h			VCAM-1	Expression	SV	
Swiss-Webster mice	Ag	7 d			Leukocytes	Expression[2]	SL[3]	[143]
	90 or 100 dB SPL	7 d				No expression		
	90 or 100 dB SPL + Ag	7 d				No expression		
	118 dB SPL	4 h	Activation	Ⅰ, Ⅱ, Ⅳ				
		7 d	No activation		Leukocytes	Expression[2]	LW	
					ICAM-1	Expression	Ⅱ	
	118 dB SPL + Ag	4 h	Activation	Ⅰ, Ⅱ, Ⅳ, HC, SC				
		7 d	Activation	HC, SC	Leukocytes	Expression	LW[4]	
			Activation		ICAM-1	Expression	Ⅱ, Ⅲ >> Ⅰ[4]	
C57/Bl6J mice	124 dB SPL	2 h	Activation	Ⅰ, Ⅱ, Ⅲ, Ⅳ, SV	iNOS	Most of NF-κB activated cells		[144]
		72 h	Activation	Ⅰ, Ⅱ, Ⅲ, Ⅳ, SV[5]	iNOS	Most of NF-κB activated cells[5]		
Sprague-Dawley rats	Cisplatin, *ip*	24 h	Activation	Ⅰ, Ⅱ, Ⅲ, Ⅳ, SV, OHC, Lim.	IL-1β	Expression	Ⅱ, Ⅳ >> Ⅰ, Ⅲ, SMV	[145]
					IL-6	Expression	SMV	
					TNF-α	Expression	Ⅰ, Ⅱ, Ⅲ, Ⅳ, SV, Lim., SMV, HC	
	Cisplatin + TNF-α inhibitor		No activation		IL-1β	No expression, no transcription		
					IL-6	No expression, no transcription		
					TNF-α	No expression, no transcription		
Sprague-Dawley rats	124 dB SPL	3 h			IL-6	Expression	Ⅲ, Ⅳ	[146]
		6 h				Expression	Ⅰ, Ⅱ, Ⅲ, Ⅳ	
		12 h				Expression	Ⅰ, Ⅱ, Ⅲ, Ⅳ, SV, SG	
		24 h				Expression	Ⅰ, Ⅱ, Ⅲ, Ⅳ, SG[6]	

[1]The time in the parentheses is the time of the maximum up-regulation of a factor; [2]The paper did not show whether it was significant or not in only the lateral wall, although it was significant in the total number of leukocytes in the modiolus, spiral limbus (Lim.), spiral ligament (SL), stria vascularis (SV), and the scala tympani. However, the number in the lateral wall showed a clear tendency of increase compared with controls; [3]There were a small number of leukocytes in the spiral ligament; [4]The leukocyte number and the intercellular adhesion molecule 1 (ICAM-1) expression intensity were significantly more than those of Ag alone and 118 dB noise alone in the total of modiolus, Lim., SL, SV, and the scala tympani; [5]The intensity at 72 h was weaker than at 2 h; [6]The intensity at 24 h was much weaker than at 12 h in the lateral wall. Some papers demonstrated nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activation in the lateral wall and cytokine expression. The systemic stressors that induce stress response of the lateral wall are shown in the column "Stressor". Each paper used different methods and time points to observe the response. For example, the activation of NF-κB (Activation) (*i.e.*, translocation of NF-κB from the cytoplasm into the nucleus), its transcriptional up-regulation (Transcription), and the protein expression (Expression) were analyzed. Some papers demonstrated which types of cells responded to stressors in the lateral wall, but some did not. Noise was band noise for 2 h in all experiments, and the intensities are shown in the table. Ⅰ-Ⅳ, type Ⅰ-Ⅳ fibrocytes in the lateral wall; Ⅱ >> Ⅰ, The NF-κB activation was much stronger in type Ⅱ fibrocytes than type Ⅰ fibrocytes; Ag, An adaptive inner ear immune response was created by sensitizing mice to the keyhole limpet hemocyanin. Then, the mice were sensitized systemically to the antigen in experimental conditions shown as Ag or + Ag in the table. iNOS: Inducible nitric oxide synthase; *ip*: Intraperitoneal injection; LPS: Lipopolysaccharide; LW: The transcriptional up-regulation or the protein expression was observed in the lateral wall: but the specific cell type was not determined; SC: Supporting cell; SMV: Spiral modiolar vein; VCAM-1: Vascular cell adhesion molecule 1; IL: Interleukin; TNF-α: Tumor necrosis factor α; HC: Hair cell; OHC: Outer hair cell.

intensity, and/or the genetic background of animal.

Adams *et al*[141] demonstrated that NF-κB of type I fibrocytes was mainly activated by an octave-band noise (90-112 dB SPL) exposure using CBA/J (Figure 1B). Masuda *et al*[144] applied noise more than two orders of magnitude greater (124 dB SPL), and demonstrated that NF-κB of the whole lateral wall was activated using C57/Bl6J mice. Miyao *et al*[143] demonstrated that NF-κB of

type Ⅰ, Ⅱ, and Ⅳ fibrocytes was activated by octave-band noise (118 dB SPL) exposure using Swiss-Webster mice. These results suggest that the same kind of stressor at different intensities or with different genetic backgrounds activates NF-κB of different regions in the lateral wall.

Different kinds of stressors also cause the different regional activation of NF-κB. As mentioned above, noise first induces NF-κB activation of type Ⅰ fibrocytes in the CBA/Bl6J mice. However, systemic inflammatory stress by peritoneal injection of LPS, a Gram-negative bacterial component, induces the activation in type Ⅱ fibrocytes with little activation in type Ⅰ fibrocytes in mice of the same genetic background (Figure 1A)[76,141]. Systemic TNF secretion by intraperitoneal anti-CD3 or taxol injection induces the same NF-κB activation as that by LPS[141]. In another report, intraperitoneal administration of cisplatin induces NF-κB activation in the whole lateral wall (Figure 1C), and this activation was inhibited by TNF-α inhibitor[145]. These suggest that LPS and cisplatin induces NF-κB activation through TNF and/or other factors that remain to be determined.

Prompt and more intense NF-κB activation in the lateral wall responding to multiple stressors than a single stressor

NF-κB activation in the lateral wall is quick after loading a stressor. For example, activation was confirmed 2 h after noise exposure in the whole lateral wall of C57/Bl6J mice, and less but still significant activation was observed after 72 h (Table 3)[144]. Using Swiss-Webster mice, the activation was observed 4 h after noise exposure but not 7 d after[142]. With intraperitoneal LPS, taxol, or anti-CD3 injection, it was observed in the type Ⅰ fibrocytes of CBA/CaJ mice after 24 h[141].

The promptly activated NF-κB regulates expression of several inflammatory factors like IL-1β, IL-6, TNF-α, iNOS, ICAM-1, and vascular cell adhesion molecule 1 (VCAM-1). IL-1β, IL-6, and TNF-α are pro-inflammatory cytokines and they are effectors and regulators of the HPA axis and the SNS, and excess NO induced by iNOS increase results in cell death, as discussed previously. ICAM-1 and VCAM-1 are critical in mediating adhesion of leukocytes to vascular endothelial cells and transendothelial migration in a variety of acute and chronic inflammatory diseases[147,148]. They also play an essential role in regulating microvascular permeability[149].

It is noteworthy that multiple stressors enhance and prolong the NF-κB activation and the target gene expression, as compared with a single stressor. Miyao *et al*[143] demonstrated that noise-exposure plus intrathecal antigen injection induced longer NF-κB activation, much more intense and wider regional ICAM-1 expression, and more leukocytes induction in the lateral wall than noise-exposure alone or antigen injection alone. The NF-κB activation was observed 4 h after but not 7 d after noise-exposure alone. On the other hand, with noise-exposure plus antigen challenge, the activation was observed even 7 d after.

STRESS RESPONSE THEORY

The original stress response theory of ISHL

There is an anecdotal hypothesis about the onset of ISHL, in which so-called "stress" (*i.e.,* psychological and physical stressors) may be associated with the onset of ISHL. It is reported that fatigue, stressful life events, inability to cope with stress, and shortness of sleep are involved in the onset of ISHL[150-152]. However, this hypothesis has a contradictory survey, as the other hypotheses do (Table 1 and see Merchant *et al*[153]). According to a survey by Japanese Ministry of Health, Labor and Welfare in 1975, rates of ISHL patients complaining of psychological and physical stress were unexpectedly low, 13.7% and 22.5%, respectively. This may suggest that a subjective scale of stress is different among individuals and it is difficult to analyze individual stress just by questionnaires.

Concerning the viral hypothesis, many reports could not show histopathological and biomolecular evidences of viral invasion or infection of the inner ear. With respect to the vascular hypothesis, it alone is not enough to explain the clinical characteristics of ISHL. For example, ISHL is not necessarily more prevalent in the elderly, does not accompany other vascular disease, and does not generally recur, making it very different from the cerebral ischemia. Furthermore, only two of 29 ears with ISHL examined showed histopathological evidence of vascular insult to the cochlea, consisting of deposition of connective tissue and new bone within the cochlea.

Finally, Merchant *et al*[53] and Adams *et al*[76,141] proposed that the stress response of the lateral wall to systemic stress is the cause of ISHL. They observed the inner ear of a patient who died 9 d after the onset of ISHL. In the affected cochlea, the organ of Corti showed marked swelling with edema, vacuole formation within the cytoplasm, and blurring of cell boundaries. They interpreted this as evidence that the cells in the organ of Corti were under severe osmotic stress, which must have resulted from lymphatic homeostasis disruption in the cochlea. In their paper published in 2005, they speculated that osmotic stress-induced NF-κB activation within the supporting cells may be an important mechanism causing ISHL in addition to the activation in the lateral wall[53]. However, using a sophisticated animal model in 2009, they demonstrated that cells of the organ of Corti and spiral ganglion were remarkable for the lack of NF-κB activation by systemic inflammatory stress[141]. On the other hand, type Ⅱ fibrocytes in the lateral wall predominantly showed the activation. The lateral wall plays an essential role in maintaining the cochlear homeostasis. In addition, NF-κB is a well-known transcription factor that directly leads to inflammatory cytokine production, and it was observed in animal and human lateral walls, but not in the organ of Corti. Conclusively, the original hypothesis by Adams *et al*[141] is that ISHL is the result of the stress response of the cochlear lateral wall through NF-κB activation responding to the systemic stress and dysfunction of the lateral wall, and

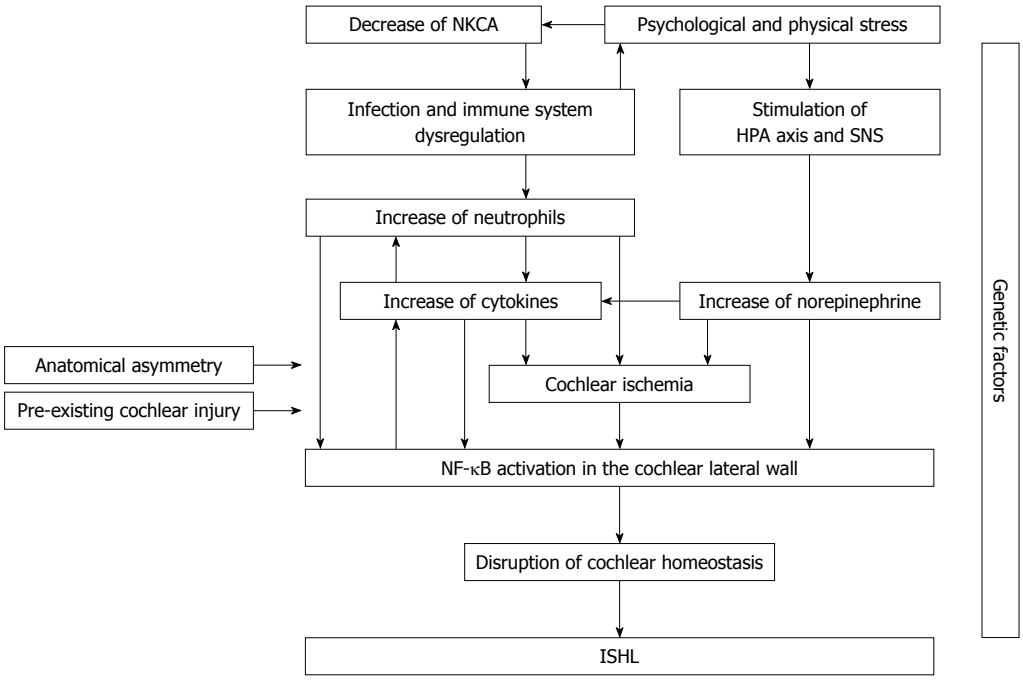

Figure 2 The stress response theory. Synchronism of different types of stressors activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) of the cochlear lateral wall and cause idiopathic sudden sensorineural hearing loss (ISHL). The causative factors should occur in order to make an effective positive feedback loop for breaking open the abnormal NF-κB activation in the lateral wall. Stressful life-events decrease natural killer cell activity (NKCA), stimulate the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS), and increase cytokines. The decrease of NKCA induces subclinical infection and/or immune system dysregulation, and then neutrophils and cytokines increase acutely, making the positive feedback loop. The acute increase of neutrophils and a cytokine like tumor necrosis factor alpha impede blood flow, resulting in acute energy failure of the lateral wall. Systemic stressors also induce and enhance norepinephrine-dependent NF-κB activation and cytokine production through the HPA axis and the SNS. They trigger the lateral wall NF-κB activation. Anatomical asymmetry, pre-existing cochlear injury, and innate factors should be involved in the flow considering the clinical characteristics of ISHL. These factors potentially explain why ISHL usually affects the ear unilaterally, why prevalence is much lower in childhood than in adulthood, and why ISHL does not affect all persons under stress. Modified from[52].

the changes of the organ of Corti cells are the secondary phenomenon to the lateral wall dysfunction.

They demonstrated that intraperitoneal LPS injection, *i.e.,* systemic stress, consistently resulted in NF-κB activation in the lateral wall unilaterally but not bilaterally, and the intratympanic LPS injection, *i.e.,* local stress, did not induce the lateral wall NF-κB activation of the mouse cochlea (Figure 1A and D). This seems to reflect the clinical characteristics of the onset of ISHL: acute onset is consistent with the prompt activation profile of NF-κB, most of cases with ISHL are unilateral, and it is not accompanied with the middle ear inflammation. They speculated that systemic cytokines like TNF-α induced by intraperitoneal LPS injection activate the lateral wall NF-κB.

However, intraperitoneal LPS injection alone activates NF-κB in the type Ⅱ fibrocytes alone and did not cause hearing loss in mice[141]. Additionally, intraperitoneal injection of anti-CD3 and taxol, which are known to induce TNF secretion, activate NF-κB in the type Ⅱ cells alone. These observations shed light on the two points: (1) a wider range of NF-κB activation in the lateral wall is needed to cause hearing loss; and (2) systemic stress by infection followed by cytokine increase alone is not enough to induce such a wide range of NF-κB activation. Therefore, the synergistic effect of multiple stressors must be necessary to induce the wide range of lateral wall NF-κB activation resulting in hearing loss.

Next, we will discuss and review how a variety of stressors including psychological and physical stressors converge in lateral wall NF-κB activation and cause ISHL.

The integrative stress response theory of ISHL

We have reviewed how psychosocial and physical stress affect the HPA axis, the SNS, the immune system, inflammatory factors, and a cytokine network. These systemic stress responses can synergistically induce and enhance lateral wall NF-κB activation (Figure 2). Although it is still impossible to demonstrate the live NF-κB activation in the human cochlea, Masuda *et al*[52] recently found evidence for the stress response theory using ISHL patients' blood sample analysis.

So-called "stress," as in chronic psychosocial and physical stress, results in EP and NEP increases through the HPA axis and SNS activation, and the cochlear lateral wall expresses these receptors (Table 2). Therefore, the stress-induced catecholamines can induce and enhance NEP-dependent NF-κB activation. Therefore, the stress-induced catecholamines can induce and enhance NEP-dependent NF-κB activation[114,154], and induce the target gene expression including pro-inflammatory cytokine, adhesion molecules, and iNOS (Table 3).

Stress decreases NKCA, resulting in dysregulation of the immune system and subclinical infections. This immune system disturbance is involved in the stress response

theory. In fact, some authors have suggested that subclinical infection is associated with onset in some ISHL patients after detecting the elevated erythrocyte sedimentation rate or specific antibodies against viruses[45,48,155].

Infection also enhances immune system dysregulation, cytokine production, and psychological stress[156]. Note that bacteria and virus do not attack the inner ear directly in the "infection" we describe here, but they change the whole immune system and have influence on the inner ear homeostasis afterward. These will lead to an increase of circulating neutrophil and cytokines like IL-6; there is a positive feedback loop between neutrophil and IL-6[157-159]. Stress also results in an abnormal immune state. Furthermore, the neutrophil increase induces cochlear energy shortage by impeding the blood flow, because neutrophils have a thrombogenic profile and are known to have association with the risk and prognosis of myocardial infarction and stroke[160-162]. Such an energy shortage induces stress response of the cochlear lateral wall.

Increase of circulating cytokines can also activate lateral wall NF-κB. IL-6 is a target and a regulator of NF-κB, and can have an impact on the NF-κB activation through classic and trans-signaling because the IL-6 receptor and gp130 are expressed in the lateral wall[163]. Circulating TNF-α activates NF-κB of the lateral wall. It also enhances microvascular tone and reduces blood flow in the cochlea[164], resulting in the lateral wall energy shortage.

The whole systemic stressors mentioned above converge synergistically to the NF-κB activation in the lateral wall. The NF-κB activation initiates inflammatory responses in the lateral wall locally. The NF-κB-induced inflammatory cytokines will affect the lateral wall cell function that maintains cochlear homeostasis. The cytokines will also exacerbate inflammatory responses of the lateral wall through enhancing vascular permeability and recruitment of leukocytes[165,166], because the blood supply to the lateral wall is abundant. In rabbits, for example, the lateral wall contains more than 80% of total cochlear blood[167]. The disruption of cochlear homeostasis ultimately causes ISHL. In fact, an ISHL-affected ear has high concentration of proteins in the inner ear fluid space using fluid-attenuated inversion recovery MRI[168-170], suggesting the disruption of cochlear homeostasis.

To explain clinical characteristics of ISHL, the integrative stress response theory should be integrated with other possible factors. At first, ISHL usually affects the unilateral ear, and the prevalence is much lower in childhood than in adulthood. Minor pre-existing subclinical damage in the inner ear or asymmetry of terminal vascular structure (for example, stenotic or not stenotic, straight or torturous) could be a potential explanation for the clinical characteristics of ISHL.

All persons under stress do not suffer from ISHL. Therefore, there must be innate factors for ISHL onset, probably including polymorphisms of genes encoding coagulation factors, vascular tone, and cytokines, among others (Table 1). Even individual personality is likely be involved in differential stress response.

Summary of the stress response theory

A quest for a single definitive cause of ISHL does not seem to be reasonable after reviewing the literature. The basic and critical concept of the stress response theory is that ISHL must not result from a specific single and local cause in the inner ear. Moreover, ISHL should encompass several causes contributing to different degrees of severity and prognosis. Synchronism of different types of factors and different degrees of contribution of each factor could result in the individual ISHL case. Some of these factors must occur rarely, and each factor must occur in a temporally appropriate order to trigger pathological NF-κB activation in the cochlear lateral wall. Therefore, ISHL does not recur frequently, even in the same individual.

Future studies about ISHL in terms of the stress response theory

We have described the possibility that psychosocial and physical stress increase the likelihood of disruption of cochlear homeostasis. Long-term stress should be detected objectively, as in HbA1c for analyzing blood sugar level over periods of 1 or 2 mo in diabetic patients. Monocyte chemotactic protein-1, epidermal growth factor, and vascular endothelial growth factor have been expected to be prolonged psychosocial stress markers[171], but the validity is still controversial[172]. There are controversies about the association of pro-inflammatory cytokines and ISHL as well[31-36,41,52] (Table 1). It may not be enough to measure and analyze the value of each biomarker separately. A new method that analyzes a complicated network consisting of multiple factors will be needed. Broderick *et al*[173] focused on the network of cytokines in which cytokine-cytokine associations are demonstrated topologically, and they demonstrated that the network of subjects with chronic fatigue syndrome deferred in topology significantly compared with healthy subjects.

Therefore, it is vital to integrate of our knowledge and comprehensive analysis of possible etiologies to reveal the pathophysiology of ISHL.

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REVIEW

Noise-induced hearing loss in the 21st century: A research and translational update

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Abstract

Millions of people worldwide are exposed to harmful levels of noise daily in their work and leisure environment. This makes noise-induced hearing loss (NIHL) a major occupational health risk globally. NIHL is the second most common form of acquired hearing loss after age-related hearing loss and is itself a major contributing factor to presbycusis. Temporary threshold shifts, once thought to be relatively harmless and recoverable, are now known to cause permanent cochlear injury leading to permanent loss of hearing sensitivity. This article reviews the current understanding of the cellular and molecular pathophysiology of NIHL with latest findings from animal models. Therapeutic approaches to protect against or to mitigate NIHL are discussed based on their proposed action against these known mechanisms of cochlear injury. Successes in identifying genes that predispose individuals to NIHL by candidate gene association studies are discussed with matched gene knockout animal models. This links to exciting developments in experimental gene therapy to replace and regenerate lost hair cells and post-noise otoprotective therapies currently being investigated in clinical trials. The aim is to provide new insights into current and projected future strategies to manage NIHL; bench to bedside treatment is foreseeable in the next 5 to 10 years.

Key words: Noise-induced hearing loss; Otoprotective strategies; Genetic susceptibility; Cochlea; Antioxidants

Core tip: Noise-induced hearing loss (NIHL) affects millions of people worldwide irrespective of age, sex, and race. Hearing aids and cochlear implants are currently the only available interventions. This review article summarizes the cellular and molecular mechanisms of NIHL to-date. Significant milestones in uncovering genetic predisposition to NIHL in humans, experimental gene therapies and post-noise otoprotective strategies to reduce the impact of NIHL are reviewed.

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INTRODUCTION

Noise-induced hearing loss (NIHL) is a major health problem indiscriminately affecting people of all ages, sex, or race worldwide[1]. A single traumatic exposure to loud sound, such as gun-shot or fireworks, or prolonged or repeated exposure to excessive sound over the acceptable daily exposure (85 dBA for 8 h, a guideline set by the National Institute for Occupational Safety and Health), cause sensorineural damage to the cochlea. This damage leads to either immediate hearing loss (impulse noise) or chronic progressive NIHL. Besides traditional hazardous exposure to occupational noise in industrial (construction, mining, forestry, aircraft, agricultural) and military set-

tings, recreational exposure is equally accountable, since many leisure activity venues (clubs, discos, gyms, sport arenas) exceed recommended sound levels. Further, the Action on Hearing Loss (United Kingdom) has issued a serious warning that approximately two-thirds of 18- to 30-year olds are exposed to dangerously high-intensity sounds (> 85 dB) which can cause hearing damage, through personal listening devices[2]. NIHL causes social isolation, impaired communication with family and co-workers, lost productivity, decreased self-esteem, depression and cognitive decline. With an aging population and the global expectation to delay retirement age, the compounding socioeconomic impact of NIHL and age-related hearing loss (ARHL) is set to become even more significant. Despite this, hearing aids and cochlear implants are the only currently available management strategies for NIHL. It is therefore crucial to develop pharmacological and molecular therapies for NIHL that can ameliorate or repair injury to the cochlea and reduce the impact of hearing loss. This paper reviews the current knowledge of the cellular and molecular mechanisms of NIHL as well as genetic predisposition to NIHL in humans and matched animal models. Significant research milestones and treatment avenues including gene therapies and post-noise otoprotective strategies achieved in recent years are discussed.

Mechanisms of noise induced cochlear injury

Sound detection by the cochlea is made possible by its sensorineural cellular elements, namely sensory hair cells and supporting cells. Outer hair cells (OHC) are electromotile and contract upon depolarization (reverse-transduction). These cells mechanically enhance the vibration of a narrow region of the basilar membrane to improve detection sensitivity (approximately 40-60 dB)[3] and frequency selectivity of the organ of Corti through cochlear amplification. The mechanical vibration is then transduced by inner hair cells (IHC), the classical sensory receptor cells, into auditory neurotransmission. This transduction is achieved through electrochemical coupling to its postsynaptic auditory afferent neurons, the spiral ganglion neurons (SGN). The structural organization of the cochlea is maintained by supporting cells lining the sensory epithelium and lateral wall tissues, the stria vascularis and spiral ligament. The supporting cells are also critical in maintaining endolymph ion homeostasis and cochlea blood supply.

The classical features of NIHL at the cellular level include damaged hair cell stereocilia, hair cell loss, swelling of afferent dendrites and SGN in Rosenthal's canal. The organ of Corti is compressed as result of damages to the supporting pillar cells, strial shrinkage, and loss of fibrocytes in the spiral limbus and spiral ligament. The cellular architecture of the high-frequency encoding basal region of the organ of Corti is more vulnerable to noise injury compared to the low-frequency apical region. This is consistent with the "half-octave shift" phenomenon[4,5] whereby the largest noise-induced threshold shifts are observed at the frequency approximately one-half octave above the stimulus frequency. This is especially true with pure tone and higher-level noise exposure, since the OHC are more prone to noise induced damage affecting their cochlear amplifier function. This sensorineural tissue damage is irreversible in the mammalian cochlea since the hair cells, which provide trophic support to the SGN, cannot regenerate. Figure 1 shows the cochlear cell types affected in NIHL.

Research using animal models of NIHL suggests two routes of cochlear damage following noise exposure. The first is that intense noise causes direct mechanical disruption of the hair cell stereocilia and direct damage to supporting and sensory cells leading to hair cell loss[6,7]. The other route is metabolic damage through various biochemical pathways that converge and cumulatively trigger hair cell death through either apoptosis or necrosis[8,9].

Current theories of metabolic damage focus on oxidative stress, which includes excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cochlea triggered by exposure to loud sound, followed by caspase-mediated cell death by apoptosis[8,10-12]. ROS have been detected in cochlear tissue immediately after noise exposure[13] and seen to persist for 7-10 d after, spreading from the basal end of the organ of Corti to the apical turn; the RNS product peroxynitrite ($ONOO^-$), generated by the combination of nitric oxide (NO) and superoxide has also been found[14]. This prolonged oxidative stress is proposed to induce the delayed and continued cochlear injury. This time might, therefore, provide a "window of opportunity" for post-noise otoprotective interventions to ameliorate or repair injury to the cochlea and reduce the impact of hearing loss. Apoptosis-inducing factor and EndoG are also released by mitochondria into the cytosol of cochlear cells following noise exposure[15]. Translocation of these pro-apoptotic factors into the nucleus triggers apoptosis. Activation of the c-Jun *N*-terminal kinase/mitogen-activated protein kinase (JNK/MAPK) signaling pathway is also implicated in OHC apoptosis in response to oxidative stress[16].

Free radicals (ROS and RNS) can cause damage by reacting with DNA, proteins, cytosolic molecules, cell surface receptors, and breaking down membrane lipids. ROS produced by the mitochondria induce lipid peroxidation in the cochlea through the formation of malondialdehyde and 4-hydroxynonenal byproducts[14]. This overloads the cochlear antioxidant enzyme system, including superoxide dismutase, catalase (CAT), glutathione peroxidase and glutathione reductase, and depletes glutathione, the endogenous antioxidant. Figure 2 provides an overview of oxidative stress pathways and the production of free radicals. In addition to apoptosis, ROS generation also leads to inflammation, and production of the pro-inflammatory cytokines interleukin-6 (IL-6)[17] and tumor necrosis factor α[18]. The presence of vasoactive lipid peroxidation products such as isoprostanes potentially also lead to the reduced cochlear blood flow associated with excessive noise[19-21]. Noise-induced ischemia and subsequent re-per-

fusion further potentiate the generation of ROS. A recent study has implicated the NO synthase/cGMP-dependent protein kinase (Prkg-1) signaling pathway, normally involved in vasodilation, in NIHL[22]. Treatment with the phosphodiesterase type 5 inhibitor vardenafil (Levitra) almost completely prevented NIHL in the rat model.

Excessive noise also leads to an increase in free Ca^{2+} in cochlear hair cells immediately post-noise[23]. This increase can be caused by Ca^{2+} entry through ion channels, such as L-type Ca^{2+} channels and $P2X_2$ ATP receptor subunit, and lead to further release of Ca^{2+} from intracellular stores[24]. Elevated Ca^{2+} levels in the cochlea may link to ROS production as well as triggering apoptotic and necrotic cell death pathways independent of ROS formation[24]. In knock-out mice lacking expression of the canonical transient receptor potential channel subtype 3 (TRPC3 channel), a non-selective cation-permeable receptor expressed in sensorineural cochlear tissue[25,26], cochlear hair cells displayed approximately 40% reduction in Ca^{2+} re-entry following intracellular calcium depletion. The TRPC knockout mice have hyperacusis at frequencies tonotopically encoded by mid-apical basilar membrane, a region highly reliant on OHC cochlear amplification[27]. The consequence of disrupted calcium homeostasis on noise susceptibility is also demonstrated in plasma membrane Ca^{2+}-ATPase isoform 2 (*Pmca2* or *Atp2b2*) mutant mice. The *C*-terminally truncated PMCA2a is the only isoform detected in the stereocilia of hair cells[28]. *Pmca2* null mice are deaf while their heterozygous littermates have significant hearing loss[29]. People carrying a homozygous mutation in cadherin 23 (*CDH23*) and a heterozygous, hypofunctional variant in *PMCA2* have exaggerated hearing loss compared to those having *CDH23* mutation alone[30].

An established mechanism of NIHL damage is the excess release of the excitatory neurotransmitter glutamate at the IHC afferent synapse. Glutamate excitotoxicity resulting from excessive glutamate release following noise overstimulation leads to an influx of cations such as Ca^{2+} across the post-synaptic membrane. The osmotic imbalance results in swelling of the postsynaptic afferent dendrites. Secondary to this cellular degeneration is calcium-dependent caspase-mediated apoptosis by intrinsic (mitochondria-mediated) pathway[31-33]. This may lead to degeneration of type 1 SGN weeks and months after a noise exposure[34]. The inhibitory neurotransmitter γ-amino butyric acid (GABA) is also associated with the regulation of auditory function[35]. Mice lacking the $GABA_{B1}$ receptor subunit have elevated hearing thresholds but increased resistance to permanent acoustic injury[35].

A theory much revisited recently is the role of intrinsic feedback pathways providing endogenous cochlear tissue protection against noise damage. Purinergic signaling through ATP activation of the ATP-gated ion channel $P2X_2$ receptor subunit within the cochlea is known to modulate cochlear function through regulating ion homeostasis[36-38]. In a recent study, Housley *et al*[39] have shown that ATP is released into the cochlear partition upon sound exposure, activating $P2X_2$ receptors, which reduce the sensitivity of the hair cells through K^+ shunting. This purinergic regulation of hearing sensitivity was revealed by the absence of noise-induced temporary threshold shift (TTS) in $P2X_2$ receptor knockout mice. $P2X_2$ receptor knockout mice also showed higher threshold shifts in response to moderate noise exposure and more substantial permanent loss of hearing sensitivity compared to their wild-type littermates, supporting the protective role of $P2X_2$ receptor signaling pathway in NIHL[40].

Noise causes psychological stress. The hypothalamic-pituitary-adrenal (HPA) axis can be activated by noise stress and directly modulate the sensitivity of the auditory system[41-43]. Glucocorticoid receptors are expressed in human and rodent cochlea[44-46]. Systemic glucocorticoids or steroid hormones are widely used to treat sudden hearing loss with variable success[47-49]. For example, dexamethasone decrease the auditory thresholds in mice subjected to a moderate acoustic trauma, while the pretreatment with glucocorticoid receptor antagonists exacerbates threshold shifts[50]. The corticotropin-releasing factor (CRF) involved in the activation of the HPA axis also modulates hearing sensitivity. CRF receptor-1 knockout mice showed elevated auditory thresholds, while CRF receptor-2 knockout mice exhibits lower auditory thresholds than wild type mice, but increased susceptibility to acoustic trauma[51,52]. Figure 3 summarizes the mechanisms of NIHL discussed.

Genetic predisposition to NIHL

NIHL is a complex condition caused by the interaction of genetic and environmental factors. Therefore, individual vulnerability to NIHL is highly variable. Understanding the genetic makeup of people susceptible to NIHL will assist in early interventions and may lead to personalized therapies. Knockout mouse studies have implicated deficits in genes involved in antioxidative pathways or the structure of the cochlea to increase susceptibility to acoustic overstimulation. These include genes encoding proteins of the hair cell (*Cdh23*[53], *Pmca2*[29]), oxidative stress (*Sod1*[54]; *Gpx1*[55]), stress-activated heat shock factor (*Hsf1*[56,57]) and potassium recycling[58,59]. In contrast, until recently little was known about the genetic factors that influence NIHL in humans. The advance in high-throughput DNA sequencing technologies, or next-generation sequencing (reviewed in Metzker[60]) has greatly accelerated understanding of human NIHL genetic predisposition. Genes shown to be associated with oxidative stress and cochlear function in mice are obvious candidate genes for human studies.

Some original linkage studies on oxidative stress genes apparently showed a link between NIHL and mutations in these genes. Glutathione S-transferase Mu 1 and theta 1 (*GSTM1* and *GSTT1*) deletion polymorphisms were found in 58 noise-exposed workers[61] and deletion polymorphisms of antioxidant genes paraoxonase 1, paraoxonase 2 and superoxide dismutase 2 (*SOD2*) were seen in

94 noise-exposed male workers[62]. However, these studies need to be interpreted with caution due to sample size and conflicting results from repeated studies with larger populations[63] (Swedish workers, 103 susceptible to noise and 114 resistant to noise). Association with the *CAT* gene was revisited by Konings *et al*[64] in two large independent populations (Swedish and Polish). In their study, additional single nucleotide polymorphisms (SNPs) were investigated to cover most of the common genetic variants. Interactions between noise exposure and genotypes and their effect on NIHL were also analyzed. Konings' study confirmed that two SNPs in CAT have associations with NIHL susceptibility, but only when noise exposure levels are taken into account. Konings *et al*[65] extended their study in the two populations and analyzed 644 SNPs in 53 candidate genes. Positive associations were shown for protocadherin 15 (*PCDH15*) and myosin 14 (*MYH14*). These are of great importance to hearing function since cadherins 23 and PCDH15 form hair cell tip links to convey force to mechanotransduction (MET) channels in sensory hair cells[66] and patients with MYH14 mutations are affected by autosomal dominant hearing impairment (DFNA4)[67].

Hair cell stereocilia are bathed in endolymph with high K^+ content, which provides the driving force for mechanosensory transduction. K^+ enters the hair cells through MET channels, exits through basolateral K^+ channels, and is recycled back to the endolymph through the outer sulcus cells, Reissner's membrane, spiral ligament and spiral limbus[68]. Mutations in genes involved in K^+ recycling, including *GJB2*, *GJB3*, *GJB6*, *KCNE1*, *KCNQ1*, and *KCNQ4* cause both syndromic and non-syndromic hearing loss (detailed in the Hereditary Hearing loss Homepage http://hereditaryhearingloss.org). Indeed, three SNPs in *KCNE1* have been shown to have significant associations with NIHL[69] and the D85N polymorphism variant, when expressed in cell culture model, showed faster channel opening and larger K^+ entry current. The same *KCNE1* SNPs and one *KCNQ4* SNP was confirmed to associate with NIHL in a later study[70].

TTS

In their seminal papers, Kujawa *et al*[71] have demonstrated that early-life exposure to noise exacerbates ARHL and that SGN are initially unharmed but dramatically degenerate 2 years after exposure to noise levels that cause TTS[34]. Their studies provided insight into the synergy between NIHL and ARHL, and also reinforced the importance of TTS in the development of progressive NIHL. A recent study of a rare heterozygous allele, *P2X2 c.178G>T* (p.V60L), presented in the DFNA41 type of progressive sensorineural hearing loss, in two unrelated large Chinese families has demonstrated neatly how environment and genetic predisposition interplay leading to NIHL[40]. DFNA41 family members heterozygous for the mutated ATP-gated $P2X_2$ receptor (*P2X2*) exhibited elevated hearing thresholds in their 20 s. Mutation carriers with history of occupational noise exposure as young adults have increased threshold shifts of 10-20 dB in the 2-8 kHz range compared to carriers with no previous noise exposure. On par with their human counterparts, *p2rx2*-null mice showed aggravated high-frequency hearing loss following continuous exposures to moderate noise from birth (8-16 kHz at 75 dB SPL). Patch-clamping and the use of fluorescent probes for membrane permeability analysis of transfected cells expressing *P2X2* p.V60L showed abolished $P2X_2$ receptor ion channel activity, suggesting impaired channel function in the mutant allele carriers. $P2X_2$ receptors are expressed in the sensory hair cells and supporting cells of the organ of Corti and the afferent SGN[38,72]. Sustained noise exposure causes up-regulation of the *p2rx2* transcripts and $P2X_2$ protein[73,74]. Noise induced ATP release into the endolymphatic compartment (the scala media) activates $P2X_2$ receptors, producing a cation shunt across the cochlear partition that reduces the driving force for both inner and OHC-mediated sound transduction[38,75,76]. The collective findings suggest the cochlear $P2X_2$ receptor/ATP-gated ion channel signaling pathway confers protection from NIHL and the absence or mutation of $P2X_2$ receptor increases susceptibility to NIHL and presbyacusis.

THERAPEUTIC STRATEGIES

Gene therapy for NIHL

Given the vital need for therapeutic options for NIHL and the known genetic influences on individual susceptibility as discussed above, gene therapy is clearly an attractive prospect. The inner ear has an anatomical advantage for gene therapy; its relative isolation in the temporal bone encapsulated in the bony labyrinth minimizes unwanted effects of the introduced gene into other tissues. Also, as a fluid-filled organ, transfection reagent can access all functionally important cells. Several different gene therapy approaches, including those focused on neurotrophic or antioxidant support and cellular regeneration, have been explored.

Neurotrophic factors and their receptors have crucial roles in the development and maintenance of SGN, and so increasing their endogenous expression by gene therapy has been widely explored to treat NIHL[77]. Experimental viral vector delivery of neurotrophic genes to the cochlea to induce endogenous expression of the gene product, including the secretion of glial cell line-derived neurotrophic factor, hepatocyte growth factor, and brain-derived neurotrophic factor (BDNF)[78], has shown promise in preserving SGN following ototoxic and noise-induced cochlear damage (reviewed in Hildebrand *et al*[79]). Alternative routes of BDNF gene delivery include the use of cochlear implants to deliver fibroblasts transduced with BDNF gene cassette giving rise to BDNF secretion[80]. In addition, the grafting of transfected BDNF-secreting NIH3T cells to the posterior semicircular canals of the adult mouse inner ear has been found to elevate BDNF production[81].

Given the importance of antioxidant enzymes in

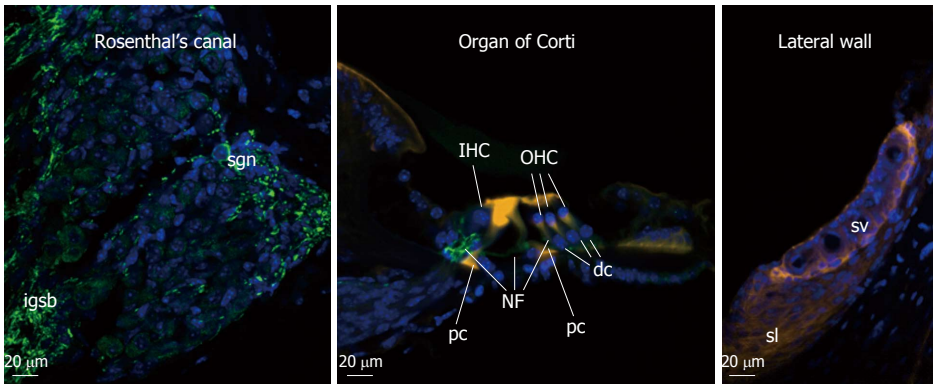

Figure 1 Cochlear cell types susceptible to noise-induced hearing loss. Fluorescence micrographs of mouse cochlea tissues by confocal scanning microscopy. Transverse cochlear section was immunolabelled to show spiral ganglion neurones and neuritis (with anti-neurofilament-F200 antibody, green), actin filament of the sensory hair cell stereocilia (Phalloidin stain, orange), and cell nuclei (DAPI, blue). In cochlea exposed to noise stress, the integrity of inner and outer hair cell (IHC and OHC) stereocilia is affected, loss of the hair cells and nerve fiber (NF), damage to supporting pillar cells (pc) and Deiters cells (dc), swelling of spiral ganglion neuron (sgn) nerve fiber (intraganglionic spiral bundle, igsb) in the Rosenthal's canal as well as loss of fibrocytes in lateral wall stria vascularis (sv) and spiral ligament (sl) can be detected.

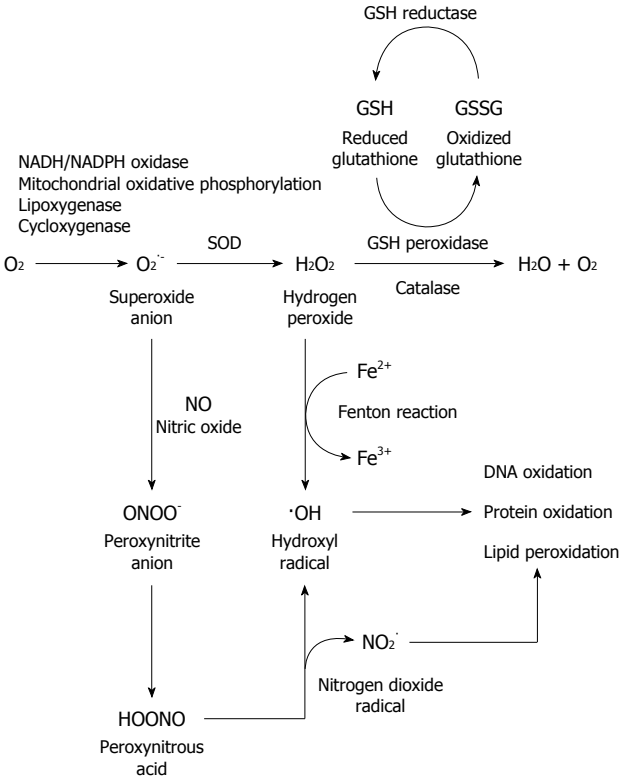

Figure 2 Mechanism of oxidative cellular damage. Mechanism of oxidative cellular damage. Reactive oxygen species (ROS, including superoxide anion, $O_2^{\cdot-}$, and hydroxyl radical, ·OH), reactive nitrogen species (RNS, nitrogen dioxide radical, $NO_2^{\cdot}$), and lipid peroxides are generated as result of oxidation of oxygen (O_2) to superoxide anion by multiple cellular oxidases. Oxidases convert oxygen to $O_2^{\cdot-}$, which is then dismutated to H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be converted to H_2O by catalase or glutathione peroxidase (GSH-Px) or to hydroxyl radical (·OH) after reaction with Fe^{2+}. In addition, $O_2^{\cdot-}$ reacts rapidly with nitric oxide (NO) to generate peroxynitrite ($OONO^-$). This further leads to production of NO_2 and cellular damages through membrane lipid peroxidation and oxidation of DNA and proteins.

curbing noise-induced free radical damage[82], gene therapy to over-express antioxidant enzymes in the cochlea may provide improved efficacy over systemic antioxidant delivery. Antioxidant gene therapy has been tested in cochlear injury induced by ototoxic drugs. Kawamoto *et al*[83] have shown a protective effect of adenovirus-mediated delivery of CAT and the SOD1 and SOD2 superoxide dismutase genes against aminoglycoside-induced cochlear injury in a guinea pig model.

Gene silencing through antisense oligonucleotides, microRNA and siRNA has been explored for otoprotection against cisplatin-induced hearing loss. Round window membrane delivery of siRNA against the transient receptor potential vanilloid 1 and transtympanic injection of siRNA against the NADPH oxidase NOX3 have shown to offer protection against cisplatin ototoxicity[84,85].

A recent advance in gene therapy is to regenerate hair cells in the adult organ of Corti. A potential strategy is to stimulate supporting cells of the organ of Corti to transdifferentiate into hair cells by the forced expression of the transcription factor Atoh1 (also known as Math 1). Izumikawa *et al*[86] showed that transfer of adenoviral vectors expressing Atoh1 resulted in the formation of "hair cell like" cells in the guinea pig organ of Corti 5 wk post-inoculation in ototoxic drug deafened cochleae. However, there are caveats in the study such as the number of new hair cells was not clear and that these cells could not be traced back to their precursors, making it difficult to delineate from hair cells that had recovered from the trauma. Further, although transdifferentiation of supporting cells to hair cells is possible, such has only been demonstrated in prenatal and neonatal preparations where both cell types are still developing[87-89]. Other studies have shown inhibition of Notch signaling to increase hair cell differentiation from stem cells in the otic placode. This mechanism is also dependent on Atoh1 activation, since silencing the transcription factor in the γ-secretase inhibitor-treated stem cells prevented the induction of hair cell fate[90]. A recent study has shown that post-noise application of a potent γ-secretase inhibitor to inhibit Notch signaling upregulates Atoh1, and leads

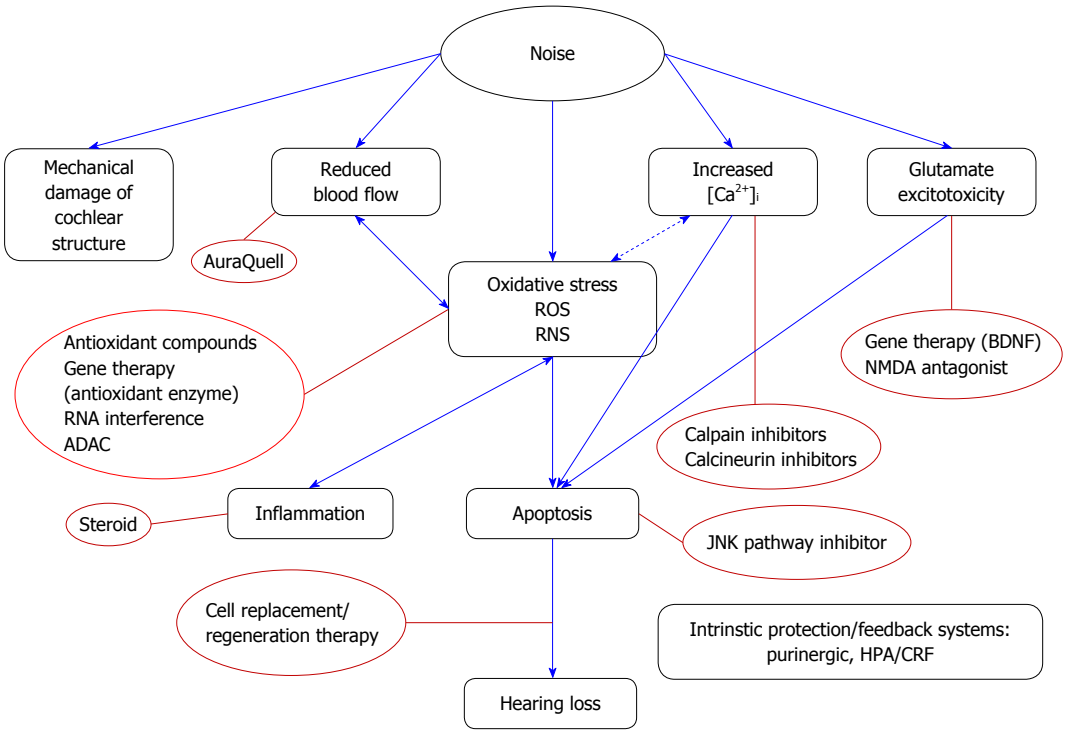

Figure 3 Overview of the mechanisms of cochlear injury in noise-induced hearing loss and interventions. HPA: Hypothalamic-pituitary-adrenal; CRF: Corticotropin-releasing factor; BDNF: Brain-derived neurotrophic factor; JNK: c-Jun *N*-terminal kinase; ADAC: Adenosine amine congener; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; NMDA: N-methyl-D-spartate.

to transdifferentiation of supporting cells into functional hair cells and improved ABR thresholds[91].

Perhaps even more innovative is the intensive research into cell-based therapy through transplantation of cells into the inner ear (reviewed by Hildebrand *et al*[79] and Shi *et al*[92]). Efforts include the generation of neurons from pluripotent embryonic stem cells and bone marrow-derived stem cells to replace or supplement auditory neurons in afferent innervation compromised by NIHL. Targeted delivery of the progenitor cells to the sensory epithelium and long-term survival and differentiation of stem cells into sensorineural cochlear tissue thus holds promise to ameliorate NIHL.

Otoprotective agents for the prevention and mitigation of NIHL

Several drugs and dietary supplements are currently in preclinical development against NIHL. The chemical structures of these otoprotective compounds are shown in Figure 4. Drug interventions in clinical trials, as depicted in the clinical database http://www.clinicaltrials.gov maintained by the National Library of Medicine at the National Institutes of Health, are included where appropriate.

Otoprotective agents against glutamate excitotoxicity, apoptosis and intracellular calcium overload

Compounds that can prevent NIHL by inhibiting glutamate excitotoxicity and apoptosis include glutamate receptor (N-methyl-D-spartate) antagonists[93] and JNK/MAPK inhibitors[16,94]. The JNK group of cytoplasmic MAPKs mediate oxidative stress-induced apoptosis and are activated by environmental stress, pro-inflammatory cytokines, and excitotoxicity[95]. CEP-1347 (KT7515) is a mixed lineage kinase (upstream regulators of MAPK kinases) inhibitor, which shows promising protection from hair cell death induced by neomycin and noise[96]. Studies using a specific inhibitor of JNK, D-JNK1-I peptide, have also demonstrated protection against NIHL and aminoglycoside-induced hair cell loss when delivered directly into the scala tympani or locally to the round window membrane of the cochlea within 24 h of noise exposure[16,94]. Developed under the name of AM-111 (Xigen/Auris Medical), a Phase 2b clinical trial has recently been completed (November, 2012) in three European countries and has shown promise for the treatment of acute sensorineural hearing loss (ClinicalTrials.gov Identifier: NCT00802425).

Direct manipulation of intracellular Ca^{2+} levels pharmacologically is not practical, but an alternative route to minimize calcium-mediated apoptosis by blocking downstream cell death pathways has been attempted. Calpain is a family of calcium-dependent cysteine proteases ubiquitously expressed in mammalian cells. Calpain immunolabeling in the cochlea is upregulated upon noise exposure, particularly in the synaptic region of the OHCs and the nerve fibers projecting to the organ of Corti[97]. Cochlear perfusion with leupeptin, a potent calpain inhibitor, prior to noise exposure reduces noise-induced hair cell loss[97]. Another drug target is calcineurin, a serine-threonine phosphatase activated by calcium-dependent calpain activation. Increased calcineurin immunoreactivity was found at the cuticular plate of hair cells immediately after noise

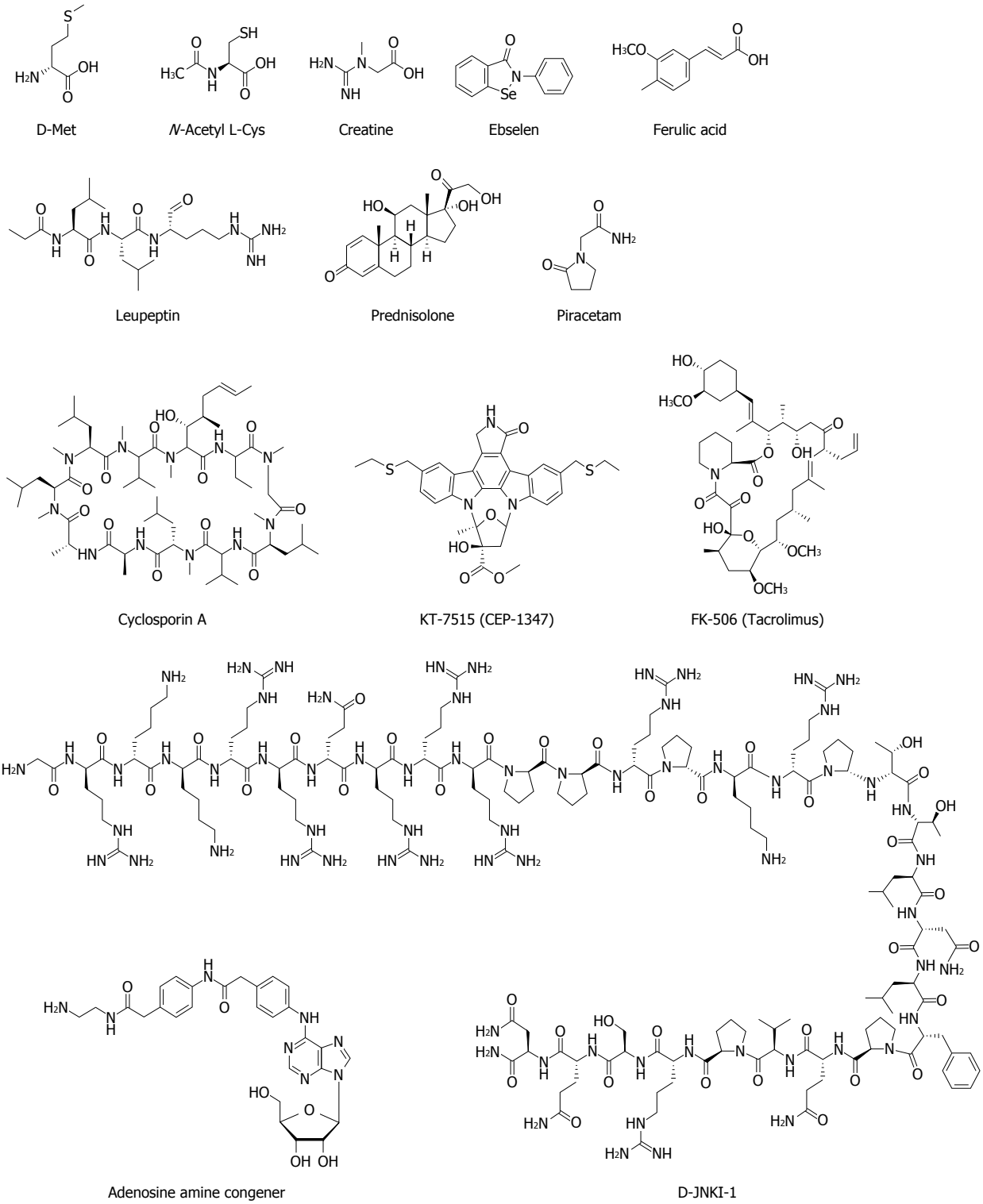

Figure 4 Chemical structures of otoprotective compounds in development or in clinical trials.

exposure[98]. Local delivery of calcineurin inhibitors cyclosporine A and FK506 to the cochlear perilymph using an osmotic mini-pump prior to and after noise exposure reduced noise-induced OHC death and hearing loss[98,99].

Otoprotective agents against noise-induced oxidative and metabolic stress

Ameliorating oxidative stress and buffering mitochondrial overproduction of free radicals is becoming an attractive avenue for the treatment of NIHL[8,100]. The potential for these therapies is highlighted by mutant mouse models. Mice with homozygous deletion of Cu/Zn superoxide dismutase 1 (*sod1* knockout)[54,101], the endogenous antioxidant enzyme that catalyzes the conversion of superoxide into oxygen and hydrogen peroxide, or homozygous deletion of glutathione peroxidase 1 (*Gpx1* knockout; the

enzyme reducing hydrogen peroxide to water)[55], have increased noise vulnerability and noise-induced hair cell loss.

All the agents aforementioned require intra-cochlear or round window administration to be effective, and most of them are used prophylactically. The surgical administration route is obviously less attractive compared to oral intake against periodic noise exposure. Therefore, orally administered antioxidant supplements with low risk of side effects constitute the majority of otoprotective therapies in preclinical development[8,82,102-104]. *N*-acetylcysteine (NAC), Ebselen, D-methionine, and ACE Mg (AuraQuell, a combination of β-carotene, vitamins C and E plus magnesium) are amongst the most studied dietary antioxidant supplements approaching different phases of clinical trials for noise injury protection. NAC is a substrate for the antioxidant glutathione synthesis, activated upon de-acetylation to L-cysteine by the liver and local tissues. It is Food and Drug Administration (FDA)-approved for respiratory disease and for reversing acute hepatoxicity following acetaminophen overdose. NAC has previously been administered either intraperitoneally, or locally through the round window membrane to prevent acute acoustic trauma[105-107]. A recent double-blind study conducted on male employee of a steel manufacturing company has found oral NAC administration to be prophylactic to TTS, particularly in subjects with susceptibility to NIHL due to their deletion polymorphism for glutathione *S*-transferases (*GSTM1* null, *GSTT1* null, and *GSTP1* Ile(105)/Ile(105))[108].

D-methionine is currently funded by the United States Department of Defense and approved by the FDA for Phase 3 clinical trial for treatment of permanent threshold shift (PTS) (Clinicaltrials.gov Identifier: NCT01345474). The amino acid D-methionine can be converted to cysteine through the intermediate homocysteine. Racemic methionine (D- and L-isoforms) is FDA-approved to acidify urine and is well tolerated when administered at doses ranging from 500 to 1000 mg/d. Like NAC, D-methionine can be administered orally, by systemic injection, or by direct application to the round window[109-112]. Ebselen is a mimic of glutathione peroxidase and has strong activity against the peroxynitrite anion ($ONOO^-$)[113]. Ebselen was protective against PTS and TTS when tested in guinea pigs and rats[114-116]. Ebselen in oral capsule (200-600 mg) is also approaching Phase 2 clinical trials for TTS (Clinicaltrials.gov Identifier: NCT01444846; Sound Pharmaceuticals).

Creatine is another dietary supplement with potential for noise-injury prevention. Catalyzed by the enzyme creatine kinase, which is present in the mitochondria, brain and muscle tissue in different isoforms, creatine and phosphocreatine engage in phosphate buffering to provide rapid regeneration of adenosine-5'-triphosphate (ATP) in tissue with high metabolic energy demand, including cochlear hair cells and stria vascularis[117,118]. The creatine transporter controls cellular availability of creatine and mutations in its gene, *SLC6A8*, lead to creatine deficiency and X-linked syndromes showing mental retardation, developmental delay, epilepsy, speech and language delay, and bilateral sensorineural hearing loss[119-121]. Creatine kinase and the creatine transporter are both expressed in the sensory hair cells, SGN, supporting cells and in the lateral wall of the organ of Corti[117,122]. A high creatine diet has been found to reduce noise-induced TTS and PTS and hair cell loss in guinea pigs[123]. Clinical trials of creatine as a single drug or adjuvant against neurodegenerative diseases (Huntington's, Parkinson's, Amyotrophic lateral sclerosis) and bipolar depression have also been carried out. These trials are based on evidence that creatine can be neuroprotective by relieving oxidative stress, and that creatine can also inhibit apoptotic neuronal death through its inhibitory action on the mitochondrial transition pore[124,125].

Otoprotective agents against inflammation and reduced blood flow

Mice exposed to noise and treated with the anti-IL-6 antibody MR16-1 show improved ABR thresholds, reduced SGN loss and a reduction in the number of activated cochlear macrophages[17]. Combined treatment with the steroid prednisolone and the nootropic drug piracetam may rescue subjects from gun-shot noise damage[126]. In spite of the lack of a control group, results look promising. A larger number of patients recovered when treatment was given within the first hour after the acute trauma compared to those receiving treatment 1-16 h after, and only 13% recovered when treatment was given after 24 h or more.

AuraQuell, developed by OtoMedicine, is a combination of antioxidant vitamins (β-carotene, and vitamins C and E) and the mineral magnesium. The magnesium acts in part as a vasodilator and in part as an antioxidant. AuraQuell is currently in Phase 2-3 clinical trial for prevention of NIHL (ClinicalTrials.gov Identifier: NCT00808470).

Compounds that provide hearing recovery after exposure to traumatic noise

Adenosine amine congener (ADAC), a selective A_1 adenosine receptor agonist, has been shown to mitigate noise-induced threshold shifts, reduce oxidative stress, and facilitate hair cell survival when applied 24 h post-exposure to noise-exposed rats (8-12 kHz band noise for 2 to 24 h at 110 dB SPL)[127]. ADAC provides neuroprotection in experimental animal models of cerebral ischemia and Huntington's disease[128-130]. Adenosine receptors are expressed in the cochlea in most cell types[131,132]. Prophylactic administration of the broadly selective A_1 adenosine receptor agonist *R*-phenylisopropyladenosine through the round window membrane can also reduce noise-induced cochlear damage[133,134], and post-exposure administration of the selective A_1 adenosine receptor agonist CCPA provides partial recovery of hearing loss[135]. Yet ADAC has advantages over other adenosine A_1 receptor agonists, as it causes minimal peripheral side effects such as bradycardia, hypotension and hypothermia, and it is able to cross

the blood-brain-barrier when applied systemically[136]. Other agents that have been shown to attenuate NIHL post exposure include D-methionine[109], ferulic acid[137], and a combination of salicylate and trolox[138].

CONCLUSION

NIHL is a preventable condition. However, even temporary hearing loss can incur cochlear injury that eventuate to permanent damage and hearing loss. Noise management and hearing loss prevention remain the principal strategies for reducing the burden of NIHL on the society and individuals. On the bright side, signifiant milestones have been reached in understanding the underlying cellular and molecular mechanisms of NIHL. The elucidation of oxidative stress as a major cause of NIHL has opened up therapeutic avenues, which was previously limited to electrical interventions of cochlear implants and hearing aids. Orally administered otoprotective compounds with antioxidant actions to protect against NIHL and "hearing pill" for post-exposure rescue will likely be available within the next decade. Advance in decoding the genetic predisposition for NIHL will facilitate early screening and will aid the development of personalized NIHL prevention and treatment strategies. Synergistically, advances in gene and stem cell therapy in animal models provide a promising path to remedy these genetic defects, and to regenerate sensory cells in the inner ear to restore hearing. These interventions would have been unthinkable until recently and these novel developments will likely change the face of NIHL research in the 21st century and reduce the impact of this sensory disability on global health.

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REVIEW

Systematic review of animal models of middle ear surgery

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Abstract

Animal models of middle ear surgery help us to explore disease processes and intervention outcomes in a manner not possible in patients. This review begins with an overview of animal models of middle ear surgery which outlines the advantages and limitations of such models. Procedures of interest include myringoplasty/tympanoplasty, mastoidectomy, ossiculoplasty, stapedectomy, and active middle ear implants. The most important issue is how well the model reflects the human response to surgery. Primates are most similar to humans with respect to anatomy; however, such studies are uncommon now due to expense and ethical issues. Conversely, small animals are easily obtained and housed, but experimental findings may not accurately represent what happens in humans. We then present a systematic review of animal models of middle ear surgery. Particular attention is paid to any distinctive anatomical features of the middle ear, the method of accessing the middle ear and the chosen outcomes. These outcomes are classified as either physiological in live animals, (*e.g.*, behavioural or electrophysiological responses), or anatomical in cadaveric animals, (*e.g.*, light or electron microscopy). Evoked physiological measures are limited by the disruption of the evoking air-conducted sound across the manipulated middle ear. The eleven identified species suitable as animal models are mouse, rat, gerbil, chinchilla, guinea pig, rabbit, cat, dog, sheep, pig and primate. Advantages and disadvantages of each species as a middle ear surgical model are outlined, and a suggested framework to aid in choosing a particular model is presented.

Key words: Otologic surgical procedures; Middle ear; Animal models; Electrophysiology; Microscopy

Core tip: Animal models provide an invaluable insight into physiological and anatomical responses to middle ear surgical interventions. The choice of which animal model to use depends on the question which needs to be answered and on the available resources. Acute terminal experiments permit greater access and exposure of the middle ear structures, but the behavioural and long-term outcomes are not available. Chronic experiments conversely have more limited options for exposure, but allow long-term observation of tissue recovery and tolerance of the surgery. Both options are considered in this review.

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REVIEW AIM

This review aims to assess the strengths and weaknesses

of different animal models of middle ear surgery by noting particular anatomical features; methods of surgical access to the middle ear; middle ear surgical outcomes, with particular attention to the hearing frequency spectrum; and advantages or disadvantages of each animal model. Unless otherwise indicated, the hearing frequency spectra are taken from work by Fay[1] and Warfield[2].

DEFINITION AND ANATOMY

Middle ear surgery is a broad term which encompasses any manipulation of the structures of the middle ear and adjoining air spaces. The human middle ear, or tympanum, is bounded laterally by the tympanic membrane (TM); medially by the promontory of the cochlea; anteriorly by the Eustachian (auditory) tube; and posteriorly by the aditus ad antrum and subsequent entry into the mastoid air cells. Superiorly is the tegmen, a bony roof on the other side of which is the middle cranial fossa; and, inferiorly is another plate of bone which separates the middle ear from the jugular fossa[3]. On the lateral aspect, the tympanic cavity is only partly bordered by the TM and so the cavity is partitioned into the mesotympanum, which is directly medial to the TM; the hypotympanum and the epitympanum (attic) which are below and above the mesotympanum respectively.

The middle ear is an air-filled space with specialised features to transmit acoustic energy from the terrestrial air environment to the fluid filled space of the inner ear. This is referred to as the middle ear transformer and in mammals consists of the malleus, incus and stapes; a chain of three articulating bones, which joins the TM to the oval window of the cochlea[4]. Several other structures of note are also present and include muscles and nerves. The tensor tympani muscle attaches to the TM, and the stapedius muscle to the neck of the stapes. The facial nerve courses along the medial wall of the tympanic cavity while the chorda tympani passes across the middle ear between the malleus and incus.

Disease processes of the middle ear manifest with symptoms of hearing impairment, otalgia (pain), otorrhoea (discharge), tinnitus, aural fullness, imbalance and vertigo due to the proximity of the vestibular system in the inner ear[5]. The aetiologies of these processes may be infective, neoplastic, traumatic, congenital, degenerative, metabolic or iatrogenic. The role of surgery in these processes is primarily to provide a safe and dry ear, and secondarily to improve hearing[6]. Other indications include access to other structures (inner ear, posterior or middle cranial fossa) or tissue biopsy[7]. In this regard the concept of a safe ear is one in which any destructive tissue such as tumour, cholesteatoma, or other nidus of infection has been removed and the middle ear is sealed off from the external environment[8].

Surgical access to the human middle ear is commonly gained either directly down the ear canal with reflection aside of the TM (transcanal), or by drilling away the mastoid air cells and entering from behind (posterior tympanotomy or facial recess approach). Any operation upon the tympanum is generally referred to as a tympanoplasty and was classically categorised by Wullstein into Types I - V, depending on how medial the reconstruction proceeded[9]. In tympanoplasty Type I the TM is patched, whereas a Type V operation requires fenestration of the inner ear due to an immobile stapes footplate. This classification has now been largely abandoned in favour of terms which describe the procedures performed in any given operation. An ossiculoplasty is any procedure which endeavours to restore sound conduction from the TM to the oval window either by direct opposition or with coupling *via* a prosthesis or tissue. Special note is made of the situation previously encountered in a Type V tympanoplasty, where the stapes footplate is immobile. Since John Shea's popularising of oval window fenestration, this procedure is referred to as a stapedectomy[10].

WHY ANIMAL MODELS?

The primary goal of modelling middle ear surgery is to improve outcomes in humans. The outcomes of interest are improvement in hearing, resolution of disease and safety of the procedure. As discussed later, there are several limitations to hearing assessment in animal models, but safety evaluation of an intervention is where models offer much utility. Even before interventions are assessed, animal models can aid in understanding disease aetiology and natural history[11-13]. While there are many differences between humans and other animals, it is possible to compare specific disease states in animals with their human counterparts through transgenic or knockout models or gene expression[14-16]. Animal models also allow researchers to investigate the impact of concomitant diseases on surgery outcomes[17]. With careful experimental design, variable extremes not possible in humans can also be studied[18,19]. If the animal has a shorter life cycle than humans, it is possible to see the effects of these diseases or interventions from many individual animals over a shorter and more practical timeframe.

Middle ear surgical animal models enable operators to gain experience, confidence and competence with procedures, thereby reducing complications from the learning curve on human patients. In stapes surgery, live animal models expose operators to complications found in human surgery. For example, a floating or depressed footplate, incus dislocation, overhanging or dehiscent facial nerve, excessive bleeding, or perilymph drainage with a dry vestibule are all possible findings[20]. Animal research explores the foundations of treatments, reinforces surgical techniques and brings advances to patient care[21].

Artificial temporal bones have been available since 1998[22] and provide an additional way for operators to refine their skills without the need for human or other temporal bones. These bones may be used where human temporal bones are unavailable[23], and offer the advantage over animal models in that they may be fashioned with equivalent dimensions to human temporal

bones. They may be constructed from synthetic resins[22], polyurethane[24], three dimensional printing with a cast powder and bonding agent[25], or selective laser powder sintering[26]. However, resin does not look or handle like bone[24], some processes leave construction materials in the finished product[26], the detail from artificial bones is less than bones from animals and may miss structures such as the chorda tympani or round window[25] or other soft tissue structures such as skin and subcutaneous tissues. Furthermore, artificial temporal bones do not model complications such as bleeding, or leaking of cerebrospinal fluid or perilymph which are possible in live animal models.

INTERVENTIONS

The scope of this review specifically pertains to middle ear surgical interventions. These include materials used for tympanoplasty and their bio-tolerance in the middle ear mucosal environment[27], as well as their impact on hearing. Such experiments include different tympanoplasty reconstruction techniques[28,29], middle ear packing[30-32], support materials[33,34], tissue glues[35-37], and active middle ear implants[38-48]. The impact of mastoid surgery has been modelled with noise[49,50], vibration[51,52], and ossicular contact[19] interventions. Examined variables include drilling duration and speed, burr size and composition (diamond or cutting). Stapedectomy is a very reproducible procedure in terms of pathology and outcomes; however, there are several variables which have been examined in animal models[53-55]. These include the degree and method of footplate removal[56-66], choice of prosthesis and graft material[67-72], and effects of tensor tympani tendon transection[20].

Complications are also able to be closely evaluated in an animal model. These include blood in the vestibule[20,69,73], and induction and effect of infection[74-77]. Noise induced injuries were not included in this review unless the noise was specifically related to middle ear surgery, such as from drilling or the blast wave and cavitation effects of the erbium-doped yttrium aluminium garnet laser[61,62]. Noise injury is distinct from, but analogous with the acoustic injury which comes from the impact of excessive surgical forces applied to the middle ear and transmitted down the ossicular chain to the cochlea[56,62,69,78]. Laser surgery avoids acoustic injury by virtue of its no-touch method, but other laser complications may include perilymph temperature elevation or direct cochlear injury[58,59,61,63,64,79,80].

OUTCOME MEASURES

The outcomes of interest in models of middle ear surgery can be broadly classified as either physiological and behavioural in live animals or anatomical in cadaveric studies. The importance of these outcome measures is to provide information on changes in middle or inner ear function as a consequence of the manipulation and which is relevant to human surgery. Measurements of changes in hearing as an outcome can be performed with behavioural tests of auditory threshold or other behavioural tests. Physiological methods, such as auditory brainstem responses (ABR), electrocochleography (ECochG) or otoacoustic emissions provide information on sound transfer through the middle ear and inner ear function, while more specific techniques, such as using laser Doppler vibrometry (LDV) gives information on the integrity of middle ear structures.

Behavioural measures of auditory threshold have an advantage that they can be more directly applied to humans, but these tests are difficult to perform, costly and require considerable specialised expertise in behavioural animal testing. The advantage of physiological methods is that they do not require any training and can be performed in anesthetised animals during or soon after the middle ear manipulation. The physiological methods, apart from LDV are also ones that can be routinely used in human ear function assessment. A limitation of all techniques, however, is the ability to differentiate the influence of the middle ear manipulation on middle ear and inner ear function using air conducted (AC) sound. When the middle ear is surgically manipulated, such as by experimental stapedectomy or ossiculoplasty, sound conduction through the middle ear will potentially change. When AC-evoked auditory thresholds are measured, the magnitude of any post-intervention conductive hearing loss (CHL) or threshold shift is confounded by a potential surgically-induced sensorineural hearing loss (SNHL). This inner ear trauma from middle ear surgery is frequently cited as a cause of SNHL in humans[81-87]. Studies which only assess AC-evoked thresholds and note a hearing loss after the intervention are therefore not able to differentiate whether this loss was due to a conductive, sensorineural or mixed pathology[54]. Some authors have attempted to define a SNHL on AC by decreased waveform amplitude or latency[88], while other authors have tried galvanic stimulation of the cochlea to bypass the middle ear[89].

The conventional method for direct stimulation of the cochlea is with a bone-conducted (BC) stimulus which bypasses the middle ear to give an indication of cochlear function. Bone conduction thresholds are routinely measured in humans using calibrated bone vibrators in audiometry, but as conventional BC transducer performance significantly deteriorates at frequencies greater than 5 kHz[90] bone-conduction thresholds are not routinely measured at higher audiometric frequencies. Measuring physiological responses with BC stimuli in smaller animals which have a higher hearing frequency spectrum, is therefore impractical and studies with BC stimuli are generally limited to the lower frequency spectrum[91]. Unfortunately it is the high-frequency basal region of the cochlea which is most susceptible to trauma, whether due to noise[92,93], chemotherapy[94] or surgical aetiologies[95]. Being unable to investigate this region significantly limits the sensitivity of BC-evoked auditory potentials to detect cochlear injury in small animals.

Because of this difficulty in accurately assessing cochlear function in small animals undergoing middle ear surgery, histopathological outcomes of cadaveric tissue have been favoured. This may involve light microscopy or transmission (TEM) or scanning electron microscopy (SEM). These outcomes have the advantage of directly assessing tissue injury, such as hair cell loss or saccule perforation, and also allow assessment of the middle ear at the same time. Biocompatibility studies of exogenous material rely on this outcome for signs of rejection, extrusion or dissolution. The disadvantages of this approach are that the animal must be euthanised to gain access to the tissue and so many animals are needed for longitudinal studies of the development and recovery of any injury.

Histology may not be a sensitive enough indicator of structural or functional injury after middle ear surgery. For example, Ikeda *et al*[66] showed that cochlear histology remained unchanged when vestibular perilymph was removed during stapedectomy, even though the endocochlear potential (EP) was substantially reduced, which would have caused a significant loss of auditory threshold as reduction in EP is strongly correlated to loss of auditory sensitivity[96].

A more recent indicator of middle ear function is LDV[43,97,98]. As with evoked auditory potentials, LDV has the advantage of being performed on live animals, but it is also able to use AC evoking sound to directly visualise TM and ossicular displacements without having to rely on the cochlea for the outcome measure[99]. While LDV is not a direct measure of auditory function, acoustic correlates of surgical interventions are possible[100].

LIMITATIONS

The key limitation with any animal model is that it is the extent of comparability to humans. Animal models only approximate humans and have their inherent limitations. Results of animal studies may not directly apply to humans due to anatomical, physiological or pathological differences between species[17,80,101]. This fallibility is inherent with all basic scientific research[102] but valuable information is still obtained when animals are exposed to the same conditions as human surgery. Tissue healing and repair of the delicate inner ear can be studied in great detail[21].

Sometimes the outcome differences between animal and human studies may be explained by anatomical differences in the middle ear, or the ossicular chain structure and its transformer properties. For example, the lever ratio of the malleus and incus in humans is significantly smaller than in cats; a human stapes weighs more than four times that of the cat; humans have a wider anterior annular ligament *vs* posterior, whereas the cat's is symmetrical; and, the rotational component of stapes motion is more prominent in humans than cats[72,103]. Removing an extensively diseased otosclerotic footplate in humans is likely to be more traumatic than removing than the mobile footplate in the catV. The anatomical differences between the squirrel monkey and human mean the tympanic cavity is much smaller in the monkey and therefore more likely to develop adhesions due to the proximity of structures[78]. Clinically, this means the results of experimental surgery in a clean and dry monkey ear may not be relevant in the human with active infection.

Occasionally, however, the differences between animals and humans are advantageous. Laser assisted middle ear surgery offers excellent results in humans[104-106] but the reported success of argon laser stapedotomy in patients contradicts the poor results anticipated by animal experimentation[64]. This was thought to be due to differences in footplate pigmentation or mineralisation, and variations of vestibular anatomy. Bellucci *et al*[21] found fibrosis invading the inner ear occurs in cats in stapes surgery, and later studies suggested it was due to invasion of the middle ear through the defect in the atticotomy. But Fee did not note any significant cases in humans and concluded this was not clinically important in human stapes surgery[107]. Foreign body giant cells were seen within 3 wk on aluminium oxide ossicular prostheses in a rabbit model, but this was not a consistent finding in human studies[108]. And the human TM can tolerate capped polyethylene prosthesis when reinforced with vein much better than in feline models[109].

Difficulties also arise when attempting to compare results from different animal models. Hydroxyapatite has been used as a substrate for crafting ossicular reconstruction prostheses, however, early animal studies produced conflicting accounts of biodegradation[110]. It was recognised that this divergence may be due to using different animal species. Audiometric differences between species have also been noted and are due to differences in configuration of the pinna and external auditory canal (EAC), the bulla size and the middle ear transformer ratio, and potentially the cross sectional area of the helicotrema at the cochlea apex[111]. Conflicting reports about the safety of cyanoacrylate tissue glues in the middle ear were in part due to different compounds being used, but even with a single compound, butyl cyanoacrylate (Histoacryl), there were different outcomes across six species used to test this glue[37].

Some species have particular anatomical features which limit their usefulness as a middle ear surgical model. The anatomy of smaller animals poses particular challenges, both in terms of access and tissue response to intervention. For example, models of myringoplasty with iatrogenic TM perforation are limited by the excellent intrinsic reparative ability of the TM[78,112]. The rat stapedial artery, a branch of the internal carotid artery, passes across the stapedial footplate and limits access to the oval window[54,113], although it is possible to cauterise this vessel and proceed[114]. Sheep have an adipose-filled and poorly pneumatised mastoid cavity which restricts a postauricular approach and cortical mastoidectomy[48,115]. Similarly, the mastoid in pigs is also poorly pneumatised and hidden by the atlanto-occipital joint[116]. For clarity, postauricular in relation to most animal models denotes a postauricular incision, soft tissue reflection, exposure and opening

of the bulla. Non-human primates are the only animals which have a similarly developed mastoid air cell system and antrum to humans[20], and postauricular may denote either the skin incision or the subsequent transmastoid approach, or both. Non-human primates also have a cartilaginous EAC which approximates the human one, although the bony canal is too long and narrow in most species for a transcanal approach to the middle ear[20]. Other specific-species limitations are further identified later in this review.

Another limitation of animal models of middle ear surgery may come from researcher inexperience with new species or techniques, which can lead to inadvertent injury. This learning curve with new species or techniques may reduce the number of research subjects. In a study of nine baboon ears, Siedentop[117] accidentally opened the horizontal semicircular canal in three ears, three ears sustained bur injury to the facial nerve, and two cochleae promontory were injured. Following a monkey death from suppurative labyrinthitis and meningitis, Hohmann *et al*[20] advocated pilot studies for researchers to become familiar with new middle ear surgical techniques. Krupala *et al*[32] and Antonelli *et al*[118] both used three animals to refine study procedures and achieve technical skill. In such pilot studies it is possible to find safe operating parameters to guide further research. In examining the effect of the CO_2 laser on the middle ear, Lyons *et al*[58] found animals which received between 20 and 30 watt laser lesions did not recover from anaesthesia. While pilot studies are useful for reducing unintended injury, Rutledge *et al*[68] acknowledged that while operative experience reduced serious complications, it does not completely eliminate them.

Experimental injury may cause unnecessary distress when the animal wakes from anaesthesia and consideration should be given to acute non-recovery experiments[72]. Operative facial nerve injury may cause facial paralysis and have animals unable to maintain adequate nutrition or eye care, and even cause death[119]. Sometimes, however, this injury is unavoidable such as in the guinea pig where the facial nerve runs posteriorly over the oval window niche and needs to be sacrificed to widely expose this area[72,120], or in rat where the stapedial artery limits access to the oval windows[54]. An insensate guinea pig or rat auricle after a postauricular incision is at risk of being bitten though social activity, so animals may need to be kept separate for several days[120]. Injury to the vestibule may result in abnormal head tilt[120], or in extreme cases poor oral intake and death. However, acute experiments prohibit behavioural outcome assessment and intervention outcome follow up over time.

SEARCH

The Medline, Cochrane and Embase databases were searched for the terms model, middle ear and surgery. The search was expanded by searching for the interventions of ossicular replacement, stapes surgery, myringoplasty, tympanoplasty, mastoidectomy and active middle ear implant. 8250 articles were then limited to animal studies and the English language. The resulting 278 titles and abstracts were reviewed for inclusion. Studies of surgical anatomy of the animal middle ear were included, but studies not pertaining to surgical intervention of the middle ear were excluded. These included interventions to induce a medical model of disease, as it was only the treatment of such diseases which were of interest. The full text of 121 articles was sought and the references of these checked for further pertinent studies. A total of 176 articles were subsequently included in this review.

MOUSE

Anatomical features

The mouse EAC is 6.25 millimetres (mm) long and has a slight rostral curve as it approaches the TM. All rodents lack mastoid air cells. The malleus and incus are firmly joined by a synarthrosis. The ossicular system is of a microtype which has restricted mobility, *vs* the freely mobile synovial joints in humans[121]. The microtype system is better for high-frequency hearing[122].

Approach

Only one study was identified which used the mouse as a model of middle ear surgery, and as there was only need to access the TM to model the mitotic activity of TM healing, the transcanal route was chosen[123].

Outcomes

The mouse hearing frequency spectrum is 1000-91000 Hz. No mouse middle ear surgical models used hearing as an outcome. The only identified study used radio-identified mitotic repair of TM[123].

Advantages

Mice are relatively cheap to acquire and house, so mouse studies can have greater numbers of subjects. Fundamentally, there are no important differences of the structure of the TM between mice and humans, except the relatively large size of the pars flaccida in mice[123].

Disadvantages

The small size of the mouse middle ear makes access and exposure challenging. Their short lifespan may complicate longer term studies by presbycusis[121].

RAT

Anatomical features

The rat facial nerve exits the temporal bone more superficially and anterorostrally than in humans. The ossicles are thinner than human ones and are almost totally hidden in the epitympanic region[119]. The stapedial artery runs between the stapes crura[119].

Approach

The cervical approach to the ventrolateral wall of the

tympanic bullae is the traditional method of accessing the middle, and inner, ear in rodents[27,88,113,124]. The post-auricular approach is relatively straightforward, provides good access and is less invasive than the ventral approach[75,77,101,119,124-126]. Similarly, the supra-auricular approach exposes the middle ear above the TM, leaving this attached to the meatal skin. This approach gives full view of the incus and its articulations and has the advantage of not having to remove bone from the bulla or cavity wall[27,127]. The transcanal route is possible, but challenging due to the dimensions of the EAC[128].

Outcomes

The rat hearing frequency spectrum is 200-76000 Hz. This was audiometrically assessed with air-conducted ABR broadband clicks[88,126]. Pathological examination included: otomicroscopy; SEM and TEM; X-ray microanalysis and bone fluorescence; haematoxylin and eosin (HE) staining, autoradiography; and methyl green-pyronin staining which clarifies the ribonucleic acid content and allows a better differentiation between living and dead cells[27,75,77,110,127,128]. Perilymph temperature elevation has also been used as an outcome measure in laser studies[61].

Advantages

The rat middle ear ventilation and drainage system is similar to humans due to the Eustachian tube, middle ear and attic communication, so the rat middle ear might have advantages over the chinchilla or guinea pig in tubal occlusion research with associate middle ear infections, as these infections occur naturally in the rat[74,76,124,129]. The rat middle ear structures are more similar to humans than for guinea pigs, with the exception of the facial nerve which is more superficial and anterorostral in rats. The rat ossicles are clearly defined and can be removed separately, as in humans[113]. It is easier to open the tympanic bulla in rats than guinea pigs due to weaker bulla joints[74]. The Long-Evans rat is not known to have hearing deficits[88]. The Fat Sand rat is found in the deserts of the Middle East and northern Africa and provides easier access to middle and inner ear anatomy due to the large bulla cavity, a thin otic capsule, and an inner ear that clearly projects into the middle ear cavity[126,130].

Disadvantages

The rat stapedial artery runs through the stapes crura, making stapes and oval window manipulations extraordinarily difficult[54,124,130]. Exposing the oval window can cause haemorrhage, cochlear injury and death[113]. Rat ossicles are nearly a quarter the size of their human counterparts and are nearly completely hidden within the epitympanum[119]. Albuquerque *et al*[74] believed the rat is harder than the guinea pig to handle as it is less docile and also the tympanic bulla was more fragile, making handling more delicate[74]. Transcanal or intact TM exposure of the middle ear was impossible unless approached ventrally[119]. Following induced middle ear infection, the bone was stronger and so opening the tympanic bulla was more difficult which in turn facilitated damage to the cochlea, vestibular system and ossicles[74]. Wistar Albino rats have hearing deficits[88]. Rat middle ear packing with Gelfoam induces osteoneogenesis and connective tissue formation with adhesions[34,125].

MONGOLIAN GERBIL

Anatomical features

The Mongolian gerbil's enlarged bulla enhances low frequency hearing sensitivity, which allows it to detect approaching predators faster[131]. The pars flaccida is circular and relatively large, 10%-20% TM surface, compared with only 2%-3% in humans[132]. The anterior mallear process extends from the anterior process of the malleus to the temporal bone, and is not seen in humans[131]. The ossicular system is of micro type, whereby the handle of the malleus is fused to the tympanic ring[121].

Approach

Postauricular[133] and transcanal[33] routes have been used for middle ear surgical models, but as these studies were chronic experiments, no comment is able to be made about the suitability of the ventral approach which is commonly used in rodents.

Outcomes

The Mongolian Gerbil hearing frequency spectrum is 100-60000 Hz, but there were no studies identified which used hearing as an outcome. Pathological specimens were examined under TEM, HE or polychrome staining and examined under the light microscope with routine and polarized light[33,133,134].

Advantages

Similar to mice and rats, gerbils are small and easy to care for. The comparatively large bulla assists in dissection and exposure, in much the same way as in the Fat Sand Rat. Gerbils are naturally relatively free of otitis media[129]. Liening *et al*[33] used the gerbil in their model because it is the least phylogenetically advanced animal known to form retraction pockets, and it does this quickly in response to Eustachian tube cautery.

Disadvantages

The researchers in these studies did not identify any specific weaknesses to using gerbils as a middle ear surgical model, however, there were few reports and the general concerns with small animal external validity apply.

CHINCHILLA

Anatomical features

Similar to the gerbil, for its size the chinchilla has a comparatively large bulla and TM[135,136]. The ossicular system is freely mobile, whereby the bones are suspended on ligaments, as they are in humans[121]. The incus and malleus are fused with cartilage, functioning as a horizontal

bar. Compared with humans, the crura are more central on the stapes footplate. A bony strut, the crista stapedis is persistent, as in the human embryo and guinea pig[136]. It passes through the stapes arch from the fallopian canal to the rim of the round window[137].

Approach

Browning *et al*[137] in their foundational work describe four surgical approaches to the labyrinth and ossicular chain. The dorsal, labyrinthine, and combined approach through the mastoid and labyrinthine parts of the bulla all go through the bulla, while the fourth approach is down EAC. No single approach demonstrates all of the middle ear, so the chosen approach is tailored to the kind of surgery required.

Outcomes

The hearing frequency spectrum of the chinchilla is 90-22800 Hz, which is remarkably similar to humans and reflects the enhanced hearing in the lower frequencies due to the proportionally large TM and bulla. Hearing measures included ABR[138], cochlear microphonics (CM)[44] and compound action potentials (CAP)[139]. The chinchilla can also be trained in behavioural audiometry[137]. Middle ear implant studies have used tone evoked CM[40,43,47], CAP and ABR[44]. The same studies also used LDV to examine stapes motion. Other outcomes of note were tympanometry and histology[140], and vestibular temperature[141].

Advantages

As noted with mice, rats and gerbils, the chinchilla is cheap to acquire and look after. The large bulla is thin and allows for easy immobilisation, middle ear access and histological preparation. After it is opened, the bulla heals firstly with fibrous tissue and then with bone so that recovery experiments are possible. The cochlea is also thin and projects into the labyrinthine bulla, so is easily accessed for electrophysiological measurements. The chinchilla's hearing is relatively susceptible to noise trauma[137]. As with gerbils, chinchillas are naturally relatively free of otitis media[129]. The life span of the chinchilla is 12 to 20 years[142], so they are useful for studies requiring normal hearing, but less good as a model of presbycusis.

Disadvantages

The chinchilla venous sinuses are within the skull bones, instead of the dura as in humans. As such, removing bone can be challenging due to the risk of haemorrhage[137]. It is difficult to identify the TM *via* the transcanal route due to the direction of the EAC, even following removal of the convoluted cartilaginous folds of the pinna[139]. The EAC runs dorsal-ventral where the lateral wall is continuous with, and in the same plane as, the lateral walls of the labyrinthine and mastoid bulla sections[137]. The chinchilla round window membrane is only one sixth the thickness as that of humans[139].

GUINEA PIG

Anatomical features

There is a foramen in the anteroinferior aspect of the EAC which resembles the foramen typanicum seen in the first few years of human life. The guinea pig TM is proportionally larger than in humans and only has *pars tensa*, above which is a bony area called the supratympanic crest. The cochlea has 3.5 turns and projects well into the middle ear, dividing it into bulla below and epitympanum above, the bulla corresponding to the meso- and hypotympanum of the human ear. The epitympanum is a slit like space which contains the fused malleoincudal complex. Compared to humans, there is a simplified air cell system of four large cells which do not have fine partitions. The oval window is orientated vertically while the round window sits horizontally. The stapes is identical to that in humans but has a bony bridge passing between the crura which is known as the crista stapedius. This structure is present in the human embryo, chinchilla and guinea pig and represents the calcified remnants of the stapedial artery. The guinea pig Eustachian tube consists entirely of cartilage. The facial nerve exits in a posterosuperior position between the tympanic ring and the bulla[54,124,136,143].

Approach

There is a large body of literature on the guinea pig as a model of middle ear surgery, and this is reflected in the number of different methods of access to the middle ear. The head is strapped to table[144] or immobilised with a custom head holder. Most authors favoured a postauricular approach to the bulla[32,45,91,118], sometimes referring to retroauricular[31,145] or postaural[146] approaches. This gives excellent access to the round window with a slight turn of the animal's head, however, the stapes is hidden by an overhanding facial nerve and a shelf of bone medial to the nerve also obscures the incudostapedial joint (ISJ). Full access to the oval window therefore requires removal of the bony shelf, sacrifice of the facial nerve and removal of the crista stapedius[54]. Special care needs to be taken of animals with facial nerve sacrifice as they are liable to develop ocular complications or feeding problems[119,120]. As a slight variant, the supra-aural approach comes down onto the epitympanum first, before exposure of the rest of the ossicular chain, cochlear promontory, and the facial nerve[28,102]. This approach was particularly useful for homograft reconstruction which required removal of the TM from its annulus[28]. The transcanal approach[30,144,147,148] is facilitated by incision of the pinna base[58] or removal entirely, however, the incision leaves the pinna insensate and it is at risk of being bitten in social activity[120]. The inferior[143] or ventral[60,73] approaches give excellent exposure, but are more suited to non-recovery anaesthesia experiments.

Outcomes

The hearing frequency spectrum of the guinea pig is 54-

50000 Hz, of which the lower half of this spectrum is commonly interrogated with AC sound. Evoked potentials include tone[79,149] and click[52,63] evoked CAP[17,54,145], tone evoked CM[73,79], and tone[31,49] and click[19,63] evoked ABR[118]. Middle ear surgical implants are also capable of evoking ABR potentials with clicks[45,46] or tones[46] *via* coupling to the ossicular chain or round window[41,45,46]. EP changes correlate with hearing impairment, and are another physiological outcome measure which indicates cochlea injury[66]. The direct current extracochlear potential has also been measured, however this does not reflect underlying function of the cochlea, rather the heating influence of a laser[150]. Other studies have also examined thermal effects in a more conventional fashion[60,79]. Postmortem analysis includes histology[32,148,151] with HE staining[146], hair cell counting[152] and SEM[50].

Advantages

The large tympanic cavity affords excellent exposure of the middle ear and the Eustachian tube is always patent[28]. This has the advantage of excellent postoperative middle ear ventilation to reduce the influence of infection or TM retraction. The facial nerve anatomy resembles that in humans, but the exit point is more superficial in the guinea pig[119]. Noise-induced hearing loss, which is thought to be analogous with surgical injury, is well established as an experimental model in the guinea pig which complements the surgical models[49]. The guinea pig is chosen in models of laser-assisted otosclerosis surgery because the basal turn of the cochlea is readily accessible and is of a similar thickness to the otosclerotic human footplate (150-200 μm)[62,63].

Disadvantages

Guinea pigs live to about 3 years. For the first half of their life ear infections are rare, but in later years they tend to have an increasing incidence of spontaneous infections[28]. Guinea pig anaesthesia is notoriously difficult for long operations with conventional techniques[28]. Such procedures benefit from a gaseous anaesthetic circuit, which increases complexity of the experiment. The fusion of the malleoincudal process limits the external validity of ossiculoplasty procedures to humans. The prominence of the cochlea is more suited to inner ear experiments[113]. Compared with rat, the guinea pig bulla is thicker and may require drilling away, whereas in rat the bulla can be gently taken apart in a piecemeal fashion[74]. Despite the relatively large bulla, simultaneous access to the oval window for interventions and the round window for ECochG may be difficult[73].

The guinea pig cochlea is more susceptible than humans to ototoxicity due to the thinner round window membrane in guinea pigs[145], and is also ten times more sensitive to acoustic trauma than humans[62]. Cochlear harm may also be overestimated in guinea pig stapes surgery because the structures most traumatised are the basilar membrane and the organ of Corti, whereas in humans it is the utricle and saccule which are most exposed[62]. Additionally, the guinea pig cochlear aqueduct is patent. This can lead to flow of cerebrospinal fluid from the subarachnoid to the perilymphatic space which may continue to leak through the oval window following stapedectomy[66]. The net effects of these features may overestimate the inner ear harm in guinea pigs from interventions.

Several authors have also noted the guinea pig possesses the ability to lay down new bone in the middle ear. Dost *et al*[120] found a control group of animals were apparently able to regrow crude stapes superstructures from the remnants of their crurae. This osteoblastic response was also seen in relation to generic middle ear bony trauma and with granulation tissue by Holzer[146]. He proposed this response was unique to the guinea pig and this notion was supported by Wells *et al*[102] who found bony overgrowth at the Histoacryl site in guinea pigs, whereas bony destruction was seen in the cat[153] and the rabbit[154], but not in the dog[155]. This finding limits the external validity of guinea pig ossiculoplasty surgery in humans, but also causes problems in the guinea pig where uncontrolled bony fixation between an implant and other middle ear structures complicated what may have been an otherwise successful operation[151].

RABBIT

Anatomical features

The rabbit bulla is larger than most mammals and has a longer EAC. The bulla is rounder and does not project past the level of the occipital bone, as found in the cat and dog[121].

Approach

The transcanal[108,156-159] route was most common and exposure was aided by a postauricular incision with reflection forward of the pinna and incision through the cartilaginous EAC, before raising the tympanomeatal flap[154]. While the postauricular incision was used for pinna control, the postauricular approach through the bulla was only advocated in one study which compared it to a human atticoantrostomy[157]. Another group did, however, advocate a combined transcanal and postauricular approach[160]. The ventral approach once again was better suited to non-recovery experiments[161].

Outcomes

The hearing frequency spectrum of the rabbit is 360-42000 Hz. Stieve *et al*[158] attempted to get around the problem of ossiculoplasty disrupting the AC stimulus by using BC click-evoked ABR, however, no comment was made as to the frequency response of transducer. Manual positioning of transducer may also provide inconsistent pressure and therefore energy transfer of stimulus. Another publication from the same group used click and 8 kHz pip stimulus ABR, traditional and SEM, but did not report their audiological findings[108]. In addition to SEM[162]; Giemsa[162,163], HE[157,159], von Gieson's stain and fibroblast growth factor stains[159] were also used with transmission and fluorescent light microscopy[164].

Advantages

As with rodents, the rabbit is cheap and easy to obtain[165], but being larger, the ear is much easier to examine perioperatively[166]. Rabbits provide a close model of human middle ear anatomy and have a standardised surgical approach[108,158]. Their response to middle ear surgery shares the same bone remodelling processes as in humans[166] without showing the ossicular osteogenic response seen in guinea pigs[156]. The anaesthesia depth can be better controlled with inhalational techniques rather than barbiturates which enabled mortality to be considerably reduced[158]. This was, however, complicated by bradycardia on endotracheal tube insertion and associated cardiac arrest. The tube effect was mitigated against with Glycopyrrolate[158].

Disadvantages

Gaining access to the rabbit middle ear is still more difficult than in humans due to the small dimensions involved[158]. There are differing opinions whether the facial nerve is dehiscent in the middle ear[162] or not[158]. A dehiscent nerve is at greater risk from middle ear surgery, but would only be a problem in non-acute experiments.

CAT

Anatomical features

The middle ear cavity of the cat is separated in two by a bony septum. This has a small hole in it so that the ear functions in a similar way to the mastoid, aditus, middle ear arrangement in humans, although the cavity effect is much greater in cats[103]. While the cat TM is smaller and thinner than in humans, the structure is essentially the same. There are two collagenous bundle layers in the middle layer of the pars tensa which are oriented radially and in a circular fashion respectively. The pars flaccida middle layer does not have a specific fibre arrangement. The lateral layer of the TM is covered by the same keratinising squamous epithelium as the EAC, and medially the TM is lined by the mucosa of the middle ear cavity[123]. The pyramidal process is bony in humans but in the cat it is cartilaginous, located next to the cartilaginous rim of the TM, and referred to as the pyramidal cone to reflect these differences[167]. The cat has the ability to regenerate its stapedius tendon[20].

Approach

Access to the cat middle ear was aided by removing the pinna[35], and by suitable head immobilisation, such as ear bars and a snout clamp[103]. Once again, the postauricular route was the most popular[20,21,29,57,70,80,124,168]. This entailed extending the postauricular incision to the posterior surface of the pinna cartilage to find the bony EAC, and then drilling posterior to the EAC until the middle ear space was blue lined and could be opened with picks[71,153]. This has also been referred to as the posterosuperior[13,132], retroauricular[123], transmastoid[65] or squamomastoid approaches[68]. In a similar way it was also possible to approach from above through the attic[69,72]. Davey began with a postauricular incision and drilled down the EAC to reach the middle ear *via* an attic approach[167]. The mediolateral approach is also possible, but requires removal of the parotid gland, digastric muscle, hyoid chain, ligature and cutting of the ICA and posterior facial vein[169]. As such, along with the ventral approaches[37,161], the mediolateral approach is better suited to non-recovery experiments. The transcanal approach was not used in any identified studies due to the narrow EAC in cats, and the excellent exposure afforded by other means[71].

Outcomes

The hearing frequency spectrum of the cat is 45-64000 Hz. Cats have been trained to behaviourally respond to tones for audiometric testing. Traditionally, this involved shock avoidance techniques[19], however, as the same information can be gained electrophysiologically[169] with CM[56,72,161], such techniques are now uncommon. Active middle ear implants were evaluated on cat stapes in the early days of this technology[39]. Histopathological studies used HE staining[29,65,68-70,80,123,170], inverted-phase and SEM[171,172], or other histology[55,67,168]. Vestibular temperature response to laser has also been investigated[64].

Advantages

As with guinea pigs, there is a large body of literature on feline middle ear surgical models, and researchers often chose cats as a model to allow comparisons with earlier work[103]. Being larger than rodents, the middle ear structures are more easily accessed and manipulated[21,72], while the cat is still cheaply acquired, housed and cared for[20]. Being larger also confers an anaesthetic survival advantage, sometimes for many months after surgery[35]. The cat middle ear also resembles the human ear much closer than the guinea pig in relation to ossicular orientation and shape, facial nerve, and middle ear muscles[119]. Cats are responsive and agile, so accurate postoperative clinical observations of hearing and balance are possible[21]. Anaesthesia can be difficult to titrate in other species, but barbiturate anaesthesia in the cat eliminates middle ear muscle activity when appropriately deep, and can be used as a guide to anaesthesia adequacy[103].

Disadvantages

The middle ear structures are robustly protected deeper in the temporal bone than rodents[119] and drilling is required for access, as the epitympanum cannot be exposed by the transcanal route due to the long, narrow and convoluted EAC[71]. The EAC can also harbour a large number of bacteria, fungi and parasites[20,173]. While the ossicles are larger than rodents, they are still smaller than in humans and surgery is therefore much more challenging with cochlear injury much more likely[71]. It would also appear that the cat TM is less robust than in humans as extrusion of a polyethylene strut was a common finding in cats[109]. A patent cochlear aqueduct was not infrequently found in cats[55] and may contribute to perilymph overflow not usually found in human stapedectomy[71].

The cat was also prone to osteoneogenesis, although this was in association with mucosal injury[29] and the significant fibrosis reaction which followed[65]. When the mucosa was not traumatised, fibrosis was not a significant problem[70]. It is likely that this repair process is influenced by the small epitympanic area and associated proximity of the middle ear structures to the wall of the cavity[174].

DOG

Anatomical features

As in the cat, the cartilaginous EAC of the dog is convoluted and can be a reservoir of bacteria and parasites[20].

Approach

Only two authors were identified who utilised a canine model of middle ear surgery. Siedentop[155] accessed the middle ear through the bulla, but does not describe his approach any further. Guilford *et al*[112,175,176] describe different approaches in their three publications. They used a postauricular incision with transection of the cartilaginous EAC and a transcanal approach with removal of the skin of the posterior EAC and overhanging canal roof in a study on incus repositioning[176], what appears to be a standard transcanal approach in a study on TM perforation repair[112], and a postauricular approach with opening of the bulla in a third study[175].

Outcomes

The canine hearing frequency spectrum 67-45000 Hz, however, none of the above studies assessed hearing as an outcome. Pathological evaluation was with HE, trichrome or Weigert's elastic tissue stains[91,112,140]. Siedentop[155] also used histological evaluation, but did not elaborate further.

Advantages

In these studies, mongrel dogs were apparently easily acquired and their middle ears are bigger than those in cats.

Disadvantages

In the perforation study it was observed that iatrogenic TM perforation would generally heal spontaneously within a few weeks[112]. This limits the utility of this model in tympanoplasty when the control group can do as well, or better, than the surgical intervention group. As there is very little data on the use of dogs as a middle ear surgical model, comparisons are difficult. Dogs are larger and more energetic than cats and rodents and so require more specialised care.

SHEEP

Anatomical features

The EAC of the sheep is highly curved and projects over the pars flaccida which is triangular and smaller than the circular pars tensa[177]. This leaves part of the lateral bulla under the bony shelf of the ventral EAC. Similarly, the inferolateral bulla extends laterally under the tympanic bone and annulus[115]. As such, the hypotympanum is particularly large. The pars tensa is proportionally large compared with other animals. The sheep middle ear is morphologically equivalent to the human middle ear[48], although the size is about two thirds smaller[115]. The long process of the incus is shorter, thicker, and closer to the body of the malleus[115]. As with the chinchilla and human, the ossicular system is freely mobile, being suspended by collagenous fibres or mucosal folds carrying blood vessels and nerves. The malleus head articular surface forms a diarthrosis with the incus body, whereas the ISJ is similar to the enarthrosis seen in humans[177]. Human and sheep round windows are similar, but sheep have no mastoid antrum and the mastoid cells are filled by adipose[177].

Approach

The only studies identified explored the potential of using sheep as a middle ear surgical model. Based on these studies, the postauricular approach was not recommended due to mastoid adipose[115], however, the transcanal route provides adequate exposure[53,115].

Outcomes

The sheep hearing frequency spectrum is 100-30000 Hz, however, no identified studies used hearing outcomes in sheep. The outcomes from stapedectomy training models focused on successful placement of the prosthesis, duration of procedure, and complications[53,115].

Advantages

Sheep easily managed in a laboratory as they do not need special care in confinement and are docile animals. They are more expensive to acquire than rodents, but tolerate surgical procedures well and have a longer lifespan. Sheep are specifically bred for human consumption so there is less objection from animal rights organisations to their involvement in research[178]. The smaller attic allows the ossicles to be accessed in the same axis as the EAC down the transcanal route, so there is no need to open the dorsal bone of the head[177]. Such a model is advocated for myringotomy, tympanotomy, ossiculoplasty and stapedectomy[53].

Disadvantages

The postauricular approach and mastoidectomy are precluded by mastoid adipose[48,53,115]. The facial nerve is thicker than in humans and often dehiscent in the tympanic cavity[115].

PIG

Anatomical features

The atlanto-occipital joint is faces posteriorly and partially overlaps the mastoid[179]. The mastoidectomy landmarks of the temporal line and suprameatal spine are not seen, and the pneumatised mastoid air cells are not found, rather there are pneumatised air cells inferior to

the tympanic cavity[180]. The external ear canal of the pig is very long and orientated posterosuperiorly[179]. The body of the incus is shorter than in humans and has another process, perpendicular to the short process[179]. The remaining ossicles are approximately the same size as their human counterparts.

Approach

As with the sheep, the only studies identified explored the potential of using pigs as a middle ear surgical model. Based on these studies, the postauricular approach was not recommended due to the overhanging atlanto-occipital joint and lack of mastoid pneumatization. The transcanal technique requires drilling to enlarge the narrow EAC, modelling the human canalplasty[179].

Outcomes

The pig hearing frequency spectrum is 42-40000 Hz[181], and due to the small volume of literature is yet to be assessed as an outcome of middle ear surgery in this species.

Advantages

While the external ear is conspicuously different to humans, the middle ear is very similar, both in terms of structure dimensions and position[179,182].

Disadvantages

The pig temporal bone has a very different appearance to the human one, which leads to increased difficulty with middle ear exposure[179]. The temporal bone also has a significant amount of soft tissue coverage and mastoid adipose[48].

PRIMATE

Anatomical features

The squirrel monkey has a straight EAC[35], the bony portion of which is very short as it consists of only an exaggerated bony annulus[78]. The temporalis muscle is large and permits fashioning of a rotation flap[78]. As noted earlier, primates are the only animals which have mastoid air cell system and antrum which are similar to humans[20].

The cynomolgus monkey has a cartilaginous EAC which resembles the human, but the bony EAC is too long and narrow to permit the transcanal approach[20].

The baboon EAC is short and narrow with a diameter of less than 4 mm and length of approximately 25 mm[183].

Approach

The straight EAC facilitated the transcanal route similar to that used in humans, either with[35] or without[184] endaural releasing incisions. Paparella[78] initially used a postauricular incision in the squirrel monkey, but abandoned this because of the required length of incision and associated postoperative wound infection. They too moved to the transcanal approach with endaural incisions and the operation was well tolerated. The postauricular approach was, however, favoured for the cynomolgus monkey[20] and baboon[117] with drilling down of the posterior EAC wall[183].

Outcomes

The hearing frequency spectrums for the squirrel and cynomolgus monkeys are 100-43000 and 28-42000 Hz, respectively[185]. Pure tone auditory thresholds were assessed in squirrel monkeys with behavioural conditioning using the shock avoidance technique while employing a double grill box[78]. This study noted hearing losses of 30 to 40 dB and assumed this was a conductive loss as the inner ears were normal on histological examination. Hardcastle *et al*[186] also assumed hearing losses to be conductive in nature due to normal cochlea histology. Lima *et al*[184] assessed AC and BC thresholds up to 16 kHz with ABR and shock avoidance behavioural testing, but they make no comment about BC masking and the BC transducer they used was the Radioear B-70A bone vibrator, which artificial mastoid testing demonstrates a poor response for frequencies above 5 kHz[90].

The single cynomolgus monkey and 18 baboons only underwent histopathological review[20,117,183].

Advantages

In the squirrel monkey, the transcanal approach is straightforward and is the preferred method of middle ear exposure[20,78]. The squirrel monkey is small and manageable and can survive for extended periods after surgery[35]. It can be readily conditioned so that pure tone auditory thresholds can be established even more quickly than in cats.

Old World monkeys, including baboons, are phylogenetically closer to humans than most other living primates, the closest being anthropoid apes[183]. Results from baboons are expected to stand the best chance of being transferable to the human middle ear. Paparella[78] also notes that the phylogenetic proximity of the squirrel monkey to man may increase the external validity of experimental findings. The same may be said for the phylogenetic closeness of the cynomolgus monkey[20].

Disadvantages

The mastoid cavity is shallow so the semicircular canals may be inadvertently exposed and opened during surgery[78]. The TM possesses a remarkable ability to repair itself which makes myringoplasty modelling difficult as even large lesions heal spontaneously within a week[78]. The cynomolgus monkey has a weak annular ligament which made it difficult to remove the stapes crura and not the footplate[20]. The baboon transverse and sigmoid sinuses are close to the posterior EAC wall, which reduces the space for surgical manipulation. The horizontal semicircular canal and the facial ridge limit visibility of the stapes footplate[183], but the stapes head and some of the crura could usually be seen[117].

CONCLUSION

The choice of animal model to use when evaluating a

Table 1 Advantages and disadvantages of each animal model of middle ear surgery

Species	Advantages	Disadvantages
Mouse	Inexpensive to acquire and house. Genetic models of hearing loss	Small size makes access and exposure challenging
Rat	Relatively inexpensive to acquire and house. Anatomy bigger than mouse	Stapedial artery. Less docile than guinea pig
Mongolian Gerbil	Small and easy to care for. Comparatively large bulla. Naturally relatively free of otitis media. Able to form retraction pockets	Few published middle ear surgical models
Chinchilla	Inexpensive to look after. Large, thin bulla. Similar hearing frequency range to humans. Naturally relatively free of otitis media. Long lifespan	Venous sinuses within skull bones. Difficulty identifying the tympanic membrane *via* the transcanal route
Guinea Pig	Well established middle ear surgery and noise literature. Large tympanic cavity. Docile and easy to handle. Bigger than rodents	Middle ear infection common with age. Challenging anaesthesia. Fused incudomalleal complex. Osteoneogenesis after trauma
Rabbit	Larger anatomy than rodents. Close model of human middle ear anatomy and response to trauma	Challenging anaesthesia. Facial nerve frequently dehiscent
Cat	Well established middle ear surgery and noise literature. Anaesthesia tolerance. Behavioural outcomes easy to assess	Compared to rodents, drilling required to access structures. Many bacteria, fungi and parasites in ear canal. Patent cochlea aqueduct
Dog	Larger anatomy than cats and rodents. Behavioural outcomes easy to assess	Few published middle ear surgical models. More energetic animals require specialised care
Sheep	Docile animals. Easy to care for. Tolerate surgical procedures well. Long lifespan. Ossicles easily accessed *via* the transcanal route	More expensive than rodents. Mastoid adipose. Frequently dehiscent facial nerve thicker than humans
Pig	The middle ear is very similar, both in terms of structure dimensions and position to humans	Difficult middle ear access due to very different temporal bone with significant soft tissue coverage and mastoid adipose
Primate	Phylogenetically closest group of animals to humans. Mastoid air cell system and antrum present	Difficult to acquire and house. Ethical objections to research. Shallow mastoid cavity

middle ear surgical intervention depends on two key factors, the goals of the experiment and the local resources available. Table 1 summarises some of the key advantages and disadvantages of the species investigated.

The research question must clearly identify what the outcome measures are and what method is required to achieve this outcome. If only a few animals are needed to answer the research question, then larger animals may be considered as a model as they are likely to model the human response more closely. There are specific limitations identified for many of the above species which need to be considered when selecting an animal model. For example, if the stapedial artery cannot be worked around, then the rat should not be selected. Larger animals tend to be more robust and tolerate surgery and anaesthesia better than small animals, but the effect of bolus intraperitoneal anaesthesia may be less predictable in a larger animal and so gaseous anaesthesia may be required.

The experimental outcomes may be histopathological, functional or both. Either outcome may be assessed with acute or chronic experiments, but where chronic experiments permit observation over time, acute experiments have the ability to sacrifice key structures which impede surgical access. The ventral approach is more suited to acute experiments as it provides excellent exposure of the middle ear in most species, but this wider exposure is at the cost of greater tissue sacrifice which may not be compatible with animal survival beyond anaesthesia. Another limitation of the ventral approach model is that there is no equivalent approach in humans because of the destructive access. The postauricular approach is suited to most chronic experiments and provides adequate exposure of the middle ear, but is not possible in the sheep or pig due to the adipose filled mastoid and overhanging atlanto-occipital joint respectively[48,53,179]. The transcanal route provides the least invasive but technically most challenging exposure of the middle ear due to the narrow confines of the EAC and the limited visibility and access this provides to the middle ear. This is obviously an issue for smaller animals such as rodents, but even primates have a narrow bony portion of the EAC. Exposure may be enhanced by widening the EAC with a drill and both the postauricular and especially the transcanal approaches are facilitated by amputation of the pinna.

Middle ear surgery which manipulates the sound conducting apparatus limits audiometric outcomes by introducing a variable CHL. By itself this would not be a problem, however, middle ear surgery is also associated with SNHL from inner ear injury[8,187,188] and so it may be impossible to distinguish the cause of a hearing loss with conventional techniques. This is an issue with any model of hearing reconstruction surgery and may be overcome by direct stimulation of the cochlea electrically[89,189,190] or with BC[91]. No identified studies used galvanic stimulation in animals, and BC is problematic in animal models because of the mismatch between the frequency output capability of the transducer and the hearing frequency spectrum of the animal. There was no artificial mastoid for calibration of BC identified in animals, but when a human artificial mastoid was used there was a significantly sharp deterioration in transducer performance above 4 kHz[90,191]. This frequency response limit is satisfactory for assessing low frequency hearing structures located towards the cochlea apex, but the higher frequencies in the basal turn of the cochlea will be missed. Of the common animal models identified in this review, the upper hearing limits for guinea pig, cat and rat are 50, 64, and 76 kHz respectively[1,2]. Further compounding this problem is that injury from middle ear surgery is focussed on hearing in the basal turn[81,192-194].

The second significant consideration when choosing an animal model of middle ear surgery are the available resources. The choice of animal will be restricted by the animals available. While primates are the closest model of human middle ear anatomy, they are expensive to acquire and care for and legislation may significantly limit the kind of study permissible. Society may also deem certain species undesirable as research subjects. Research with the chinchilla and gerbil models are limited to places in the world where they occur naturally, as any advantage in importing the animal is likely to be offset by the cost differential with locally available rats or guinea pigs. Local facilities may also limit what kinds of animals are able to be cared for, and whether the animal unit has previous experience with that species. Anaesthetic modality may also influence the types of research able to be supported, as not all laboratories will have gaseous anaesthesia or perioperative support services. Choice of model is also influenced by the human resources available and experimenter experience as well.

This review has identified a significant body of evidence for tolerance of novel materials (packing, oval window graft, prostheses), and laser surgery, most of which has utilised histological rather than functional outcomes. Few studies were found which actually modelled surgery and investigated hearing outcomes. This is likely to be due to the inherent difficulty in assessing cochlear reserve in small animals when the middle ear has been disturbed. Further research is needed to develop models which are compatible with any disruption caused by middle ear surgical exposure or other intervention. Nevertheless, animal research has made a significant contribution to the development of middle ear surgical techniques and brought advances to treatments and patient care.

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REVIEW

Noise-induced cochlear inflammation

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Abstract

Hearing loss is the most common sensory disability with considerable social and economic implications. According to recent World Health Organization estimates, 360 million people worldwide suffer from moderate to profound hearing loss. Exposure to excessive noise is one of the major causes of sensorineural hearing loss, secondary only to age-related hearing loss (presbyacusis). Since cochlear tissues have limited abilities of repair and regeneration, this damage can be irreversible, leading to cochlear dysfunction and permanent hearing loss. Recent studies have shown that cochlear inflammation can be induced by noise exposure and contribute to the overall pathogenesis of cochlear injury and hearing loss. The cochlea is separated from the systemic circulation by the blood-labyrinth barrier, which is physiologically similar to the blood-brain barrier of the central nervous system. Because of this feature, the cochlea was originally considered an immunologically privileged organ. However, this postulate has been challenged by the evidence of an inflammatory response in the cochlea in the presence of bacterial or viral pathogens or antigens that can cause labyrinthitis. Although the main purpose of the inflammatory reaction is to protect against invading pathogens, the inflammatory response can also cause significant bystander injury to the delicate structures of the cochlea. The cochlear inflammatory response is characterised by the generation of proinflammatory mediators (cytokines, chemokines and adhesion molecules), and the recruitment of inflammatory cells (leukocytes). Here, we present an overview of the current research on cochlear inflammation, with particular emphasis on noise-induced cochlear inflammation. We also discuss treatment strategies aimed at the suppression of inflammation, which may potentially lead to mitigation of hearing loss.

Key words: Cochlea; Inflammation; Noise; Hearing loss; Otoprotection

Core tip: Noise exposure, either occupational or recreational, is a major cause of sensorineural hearing loss in the industrialised world. Hearing loss is a devastating disability with considerable social and economic implications. Recent studies have demonstrated that an inflammatory response induced in the cochlea by noise exposure may contribute to the development of noise-induced hearing loss. Better understanding of the underlying inflammatory processes will help define pharmacological interventions that can potentially mitigate noise-induced cochlear inflammation and the associated hearing loss.

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NOISE-INDUCED HEARING LOSS

The human ear is an exquisitely sensitive organ, allowing

us to perceive and distinguish among the myriad sounds around us, be they pleasurable, informative or damaging. Located within the inner ear is the cochlea, the specialised peripheral end organ of the auditory system, which mediates the transduction of sound waves into electrical nerve impulses that travel to the brain for central processing of auditory information. Unfortunately, this extreme sensitivity of the cochlea comes at a cost as it makes it highly susceptible to injury when exposed to loud sound. The consequence of this injury is the loss of hearing, which can be either temporary or permanent. Noise-induced hearing loss may result from either brief exposure to an intense "impulse" noise or sustained and repeated exposure to excessive sound levels (*i.e.*, continued exposure to high levels of noise over an extended period of time). The hearing loss from noise exposure is typically binaural (symmetric), and the severity of it is related to the intensity, frequency, duration and temporal characteristics (*e.g.*, impulse/impact, intermittent or continuous noise) of the noise exposure[1,2].

Excessive noise is the most common occupational and environmental health hazard. Dangerous levels of noise are generated in a large number of workplaces such as construction sites, mines, saw mills, military bases, and airports, among many others. Although usually associated with occupational exposure, noise-induced hearing loss is becoming increasingly prevalent in recreational settings. Many people, especially children and teenagers, voluntarily expose themselves to potentially injurious noise levels *via* portable music players, stereos, video games, rock concerts, and nightclubs. Other non-occupational sources of loud noise include firearms, power tools such as chain saws and drills, lawn mowers, and recreational vehicles such as motorcycles.

Noise-induced hearing loss is the second most common sensorineural hearing deficit, after age-related hearing loss (presbyacusis), and is the leading cause of preventable sensorineural hearing loss (SNHL) in the industrialised world[3]. According to recent global estimates released by the World Health Organization (WHO, 2012), there are 360 million people worldwide (over 5% of the world's population) with disabling hearing loss. Disabling hearing loss, as defined by WHO, is "hearing loss greater than 40 dB in the better hearing ear in adults and a hearing loss greater than 30 dB in the better hearing ear in children". A significant proportion (16%) of the disabling hearing loss in the adult population in the world is attributed to occupational noise exposure[4]. In the United States, approximately 15% (26 million) of people between 20 to 69 years of age have high frequency hearing loss from overexposure to loud noise at work or during leisure activities. Hearing loss has considerable social and economic implications at both the individual and societal levels. This devastating sensory disability and the serious communication difficulties has a negative impact on the quality of life of the affected individual and can lead to feelings of loneliness, social isolation and depression.

Although it can be permanent and not fully treatable, noise-induced hearing loss is virtually 100% preventable. Obviously, the best preventive measure against noise-induced hearing loss is to completely avoid or minimise exposure to excessively noisy environments. When this is not possible, the only preventative measure available is the consistent and proper use of hearing protection devices such as earplugs and earmuffs. When used correctly, these protective devices can provide 20 to 40 dB of attenuation, however their use is often impractical in many settings and they are not completely effective in harsh environments, or because of incorrect use.

Avoiding or reducing modifiable risk factors associated with noise-induced hearing loss such as voluntary exposure to loud noise, non-use of hearing protection, cigarette smoking, lack of exercise, poor diet (low dietary intake of antioxidant-rich food), and poor oral health (tooth loss) may reduce the risk or delay the onset of this debilitating condition[5,6]. The presence of cardiovascular disease and diabetes are also major risk factors. In addition to these, several non-modifiable risk factors related to noise-induced hearing loss exist, particularly age and genetics[5]. Age plays the most significant role, with the risk typically increasing with advancing age. Furthermore, great genetic variability in the susceptibility to noise-induced hearing loss has been documented in both humans and mice[7].

The association between noise exposure and hearing loss was first recognised by the physician Sir Francis Bacon (1561-1626)[8]. In 1890, Habermann was the first to describe the cochlear histopathological features of noise-induced hearing loss from examining the temporal bones of an elderly ex-boilermaker[9]. However, it was not until 1907 that Wittmaack conducted the first experimental research of noise-induced deafness in animals[9,10]. Substantial insights into the pathophysiology of noise-induced cochlear injury were gained by Wittmaack's experiments and the many others that followed, including Hallowell Davis's systemic studies on guinea pigs and humans at Harvard University in 1943.

The cochlea sustains dramatic cellular injury following noise overexposure. The pathological consequences (pattern and extent) depend on the acoustic characteristics of the noise (*i.e.*, sound intensity, frequency and duration), age and genetics[10]. The two types of hearing loss from noise exposure - temporary and permanent hearing loss (also known as temporary and permanent threshold shift) - also vary in their mechanisms[11]. Noise exposure is known to produce a variety of structural changes to the various cells within the cochlea. The most vulnerable are sensory hair cells, particularly the outer hair cells, which have traditionally been the focus of most hearing loss studies. A major impact is on sensory hair cell stereocilia which can undergo mechanical damage during noise exposure. Other changes include the loss of outer hair cells, damage to the inner hair cell - auditory nerve synapse, swelling of the primary auditory neurones in the spiral ganglion, damage to the supporting cells, acute swelling of the stria vascularis, reduced cochlear blood flow and

the loss of fibrocytes in the spiral ligament[2,11-15]. In addition, direct mechanical disruption of the cochlea can be induced by impulse noise exposure, *e.g.*, rupturing of the organ of Corti and its separation from the basilar membrane.

COCHLEAR INFLAMMATION

Cochlear inflammation has been implicated as a major etiologic factor in a range of conditions that cause hearing loss. These include acoustic trauma (noise-induced cochlear damage), otitis media (middle ear infection), meningitis, autoimmune inner ear disease, and ototoxicity (drug-induced inner ear damage, *e.g.*, aminoglycoside antibiotics, platinum-based chemotherapeutic agents)[16-23]. Labyrinthitis can also be evoked by cochlear surgery and the insertion of cochlear implants[24,25]. Pathogen-induced labyrinthitis as a consequence of otitis media or meningitis is usually associated with bacterial and viral infections. Labyrinthitis secondary to otitis media (tympanogenic labyrinthitis) primarily occurs by the spread of the infection from the middle ear into the inner ear through the three-layered round window membrane[18,22,26,27]. Meningogenic labyrinthitis most likely occurs by the spread of infection from the meninges into the perilymphatic space of the cochlea through the cochlear aqueduct[19,28,29]. Mycotic (fungal) labyrinthitis is rare, and is usually associated with systemic debilitating diseases and occurs by either the tympanogenic, meningogenic or hematogenic route[30].

Labyrinthitis usually affects the cochlea more severely than the vestibular system, resulting in adverse effects on cochlear function[26]. A well-documented complication of cochlear inflammation is partial or complete SNHL. Pathological consequences that have been observed in animal models of cochlear inflammation include degeneration of hair cells of the organ of Corti, disruption of fibrocytes in the spiral ligament, loss of interdental cells of the spiral limbus, swelling of the stria vascularis, and vascular damage[26,31-33]. The disruption of the spiral ligament fibrocytes has been suggested as a major contributor to the inflammation-induced cochlear dysfunction[32,34]. Decreased immunostaining for gap junction protein connexin 26 in type I and type II fibrocytes and decreased Na^+-K^+-ATPase staining in type II fibrocytes, both of which are critical in the maintenance of cochlear homeostasis, were observed in a guinea pig model of labyrinthitis induced by inoculation of the protein antigen keyhole limpet hemocyanin (KLH) into the scala tympani[35]. In addition, reduced connexin 26 immunostaining in the spiral ligament was also demonstrated in a mouse model of otitis media induced by the transtympanic inoculation of viable *Streptococcus pneumoniae*[36].

Analogous to the central nervous system and the retina of the eye, the cochlea is separated from the systemic circulation by a blood-labyrinth barrier, which has similar physiological characteristics as the blood-brain barrier and the blood-retinal barrier. This barrier is important in maintaining the ionic composition of the cochlear fluid compartments, and is essential for the functional integrity of the cochlea[37]. Because of the existence of this blood-labyrinth barrier and the relative absence of resident tissue macrophages, the inner ear was originally considered an immunologically privileged organ, isolated from the immune system and protected from immune surveillance. However, this hypothesis has been refuted by research demonstrating that the inner ear is capable of rapidly generating an active inflammatory/immune response in the presence of antigens or pathogens. In addition, connections exist between the inner ear and the systemic lymphatic system through cervical lymph nodes[38].

Although the intended purpose of the immune response in the inner ear is to defend the hearing organ against invading pathogens and to clear cellular debris, the inflammatory response can also cause significant bystander injury to the delicate structures of the cochlea[37,39]. Because mammalian inner ear tissues have limited abilities of repair and regeneration (unlike avian auditory hair cells which have the capacity to regenerate), this damage is irreversible, leading to permanent hearing loss. Immune-related cochlear inflammation is increasingly recognised as a potential mechanism of inner ear disease and associated hearing loss. Systemic administration of immunosuppressive drugs (*e.g.*, corticosteroids) has been shown to effectively ameliorate some cases of idiopathic, rapidly progressive bilateral SNHL, implicating inner ear inflammation as an underlying mechanism of the hearing loss[40]. Histopathological studies of human temporal bones also support the hypothesis that a number of otological disorders are linked with inflammatory responses[41]. The severity of hearing impairment and the potential for recovery correlate with the extent of inflammation-induced tissue damage. Animal studies have demonstrated that the development of inflammation and hearing loss following an immunological challenge can be rapid, with the onset of hearing loss occurring at 12 to 15 h, and peaking at 24 to 48 h[42,43].

Regardless of the cause, the cochlear inflammatory response follows a similar course with three characteristic stages: an initial acute stage, a fibrotic stage, and an ossification stage[44]. The acute phase of cochlear inflammation, which lasts approximately 3 to 7 d, is characterised by the production of proinflammatory mediators such as cytokines and chemokines, an increased expression of adhesion molecules, the recruitment and infiltration of inflammatory cells such as polymorphonuclear leukocytes (mostly neutrophils), monocytes, macrophages and lymphocytes, and the breakdown of the blood-labyrinth barrier[31,44]. In the chronic stage of the cochlear inflammatory response, a fibrotic matrix is formed in the perilymphatic spaces, which later becomes calcified. This bony occlusion of the fluid-filled cochlear scalae, known as labyrinthitis ossificans, is most extensive in post-meningitis cases[45].

The cochlea itself can mount an immune response. Resident cells in the cochlea can express a range of in-

flammatory mediators, which are thought to play critical roles in the inflammatory response[46,47]. The cochlea communicates with the immune system *via* the systemic circulation. Entry of inflammatory cells occurs primarily through the spiral modiolar vein and its tributaries (collecting venules) situated at the base of the scala tympani[48]. Inflammatory cells accumulate in the perivascular space surrounding the spiral modiolar vein, and then stream into the scala tympani along the extravascular space of the collecting venules. Other areas where circulating inflammatory cells enter the cochlea include the blood vessels of the spiral ligament and the spiral ganglion. The lateral wall of the cochlea and the spiral ganglion represent the most permeable parts of the blood-labyrinth barrier, partly due to their high vascularisation[49,50].

The mammalian cochlea contains resident macrophages at normal/steady state[16,25,49,51]. These macrophages are phenotypically similar to the tissue macrophages in other organs of the body (*e.g.*, microglia of the central nervous system) and are found in small numbers predominantly in the spiral ligament and the spiral ganglion. Moreover, it was recently reported that a large number of perivascular resident macrophages (PVMs) are present in the stria vascularis surrounding the endothelial cells of the capillaries[52]. Data from radiation chimeras have shown that these resident macrophages in the cochlea form an exchanging and migratory population, supplied continuously from haematopoietic precursors in the bone marrow, and exhibiting slow turnover during steady-state conditions[25,49,52]. These haematopoietic precursors migrate into the cochlea and differentiate into tissue macrophages. Bromodeoxyuridine (BrdU) labelling has demonstrated that the marked increase in macrophage numbers in the cochlea following an insult such as noise exposure is not due to the proliferation of these resident cochlear macrophages, but rather occurs by the migration of macrophages from the vascular system[16,53].

The signals that initiate the recruitment and infiltration of inflammatory cells into the cochlea are still under scrutiny, and a wide range of soluble mediators (*e.g.*, cytokines, chemokines) may be involved. The sources of proinflammatory mediators in the cochlea include various resident cochlear cells types (*e.g.*, spiral ligament fibrocytes, supporting cells) and infiltrating leukocytes migrating from the cochlear vasculature. *In vitro* studies using cultured murine spiral ligament fibrocytes have shown that upon stimulation with proinflammatory cytokines, fibrocytes secrete a variety of inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine, soluble intercellular adhesion molecule-1 (sICAM-1) and vascular endothelial growth factor, which play important roles in the recruitment of inflammatory cells into the cochlea[32,33,47,54]. The secretion of sICAM-1 is compatible with an earlier study that reported strong intercellular adhesion molecule-1 (ICAM-1) expression in the spiral ligament and spiral modiolar vein in the early phase of labyrinthitis induced by the inoculation of KLH into the scala tympani[55]. It is speculated that chemokines produced by the fibrocytes are presented to the surface of vascular endothelial cells *via* the process of transcytosis, which consequently attracts inflammatory cells. Fibrocytes, vascular endothelial cells, and inflammatory cells together may form networks interconnected by cytokines, chemokines and various other inflammatory mediators[32,47].

It is well documented that inhibition of TNF-α with the soluble TNF-α receptor-FC fusion protein Etanercept, given either systemically or directly into the cochlea, significantly attenuates the cochlear inflammatory response[56]. This suggests that TNF-α plays a major role in the development of cochlear inflammation. Studies on organ of Corti explants have shown that TNF-α alone, in the absence of antigens or pathogens, has the ability to induce the recruitment of inflammatory cells into the cochlea from the systemic circulation[57]. TNF-α is also expressed by infiltrating leukocytes, suggesting that it is likely involved in a positive feedback loop that further amplifies the recruitment of inflammatory cells. This is supported by the evidence that TNF-α inhibition can prevent the recruitment of inflammatory cells into the cochlea[56]. TNF-α can also induce nitric oxide synthesis by stimulating the expression of inducible nitric oxide synthase (iNOS), which can further aggravate inflammation and degeneration in the cochlea[58].

The expression of many proinflammatory mediators is mostly regulated by nuclear factor κB (NF-κB)[23]. NF-κB comprises a family of inducible transcription factors that play a pivotal role in immune and inflammatory responses. Activation of NF-κB induces the transcription of cytokines such as TNF-α, IL-1β and IL-6, as well as iNOS, and the adhesion molecules, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). NF-κB activation in the cochlea has been demonstrated following intraperitoneal injection of lipopolysaccharide (LPS)[59], suggesting that the cochlea can become immunologically active even after systemic administration of bacterial toxins. Cochlear activation of NF-κB has also been reported to occur following acoustic trauma (see the following section) and in cisplatin-induced ototoxicity[21].

At present, it is technically impossible to positively identify inflammatory processes within the human inner ear. There are no well-defined detection methods available and diagnostic biopsy of the human cochlea is not feasible. To overcome this limitation, high field magnetic resonance imaging (MRI) techniques were recently developed by our group to quantitatively evaluate the development of cochlear inflammatory processes in a guinea pig model induced by the intratympanic injection of LPS[60]. For the first time, dynamic changes in cochlear vascular permeability following cochlear inflammation was quantified using dynamic contrast enhanced-MRI and ultrasmall superparamagnetic iron oxide particles were used to characterise the recruitment of macrophages into the cochlea. These methodologies therefore hold considerable poten-

tial as diagnostic tools for human inner ear diseases such as labyrinthitis and could also be used to quantitatively assess the efficacy of treatments for cochlear inflammation.

NOISE-INDUCED COCHLEAR INFLAMMATION

Recent years have advanced our understanding of the underlying mechanisms of noise-induced cochlear damage. One of the most compelling hypotheses postulates oxidative stress (the excessive formation of reactive oxygen species or free radicals) in the cochlea as a key mechanism of noise-induced hearing loss[10,14,61]. An increase in reactive oxygen species is also thought to be involved in age-related and drug-induced hearing loss (ototoxicity). Oxidative stress alters the redox balance of the cells, leading to the activation of cell death pathways (apoptosis and necrosis) in the cochlea and hearing loss.

Other studies, however, have implied the intrinsic involvement of inflammation in noise-induced cochlear tissue damage. Early ultrastructural studies in the noise-exposed mammalian cochlea have identified macrophage-like cells in the damaged organ of Corti, mainly in the tunnel of Corti and in the outer hair cell region, appearing 5 d after acoustic overstimulation[62,63]. These macrophages are likely involved in mopping up cell debris. The presence of transforming monocytes in the area and mononuclear leukocytes within the spiral lamina blood vessels suggested that these dendritic macrophages originated from blood-borne monocytes[63].

Several studies have demonstrated that after acoustic trauma, a large influx of inflammatory cells from the vasculature can be observed in the cochlea, generally peaking between 2 and 7 d after exposure to traumatic noise, and diminishing thereafter[16,17,64-66]. Inflammatory cells within the cochlea were identified immunohistochemically using their cell surface markers CD45, a receptor tyrosine phosphatase present on all hematopoietic/bone marrow-derived leukocytes or F4/80, a marker of activated macrophages and monocytes. The study by Tornabene *et al*[17] showed that CD45-positive cells increased from an average of 0.3 cells/section in the non-exposed cochlea to a maximum of 88 cells/section at 2 and 4 d after noise exposure. These infiltrating cells were localised predominantly in the spiral ligament, particularly in the inferior region among type I and type Ⅳ fibrocytes and in the region adjacent to the bony cochlear capsule among type Ⅲ fibrocytes, and in the perilymph-filled spaces of the scala tympani and scala vestibuli[16,17,64,65]. Leukocytes were also observed within the spiral limbus, another region known to be susceptible to acoustic injury, and in the spiral ganglion[16,64,65]. A few cells were also found in the stria vascularis and the perivascular spaces of the modiolus[17,67]. This recruitment of macrophages to the cochlea following excessive stimuli is similar to what occurs in other sensory organs, such as the retina of the eye. Thus, exposure to damaging light causes an infiltration of inflammatory cells to the light-damaged region of the retina[68].

BrdU labelling has demonstrated that these inflammatory cells migrate from the vasculature, and it appears that most of these cells enter the cochlea through the blood vessels of the lateral wall[16]. The lateral wall is highly vascularized, and the spiral ligament is the site where the large majority of inflammatory cells can be found. Immunostaining with other monocyte/macrophage markers (CD68, CX3CR1, Iba-1) demonstrated that the vast majority of these infiltrating cells are derived from the monocyte/macrophage lineage, with a small number representing other leukocytes such as T and B lymphocytes[16,25]. Hirose *et al*[16] coined the term "cochlear macrophage" for those inflammatory cells, to indicate an inducible exchanging population of phagocytic cells that respond to acoustic injury.

The recruitment and extravasation of these inflammatory cells into the cochlea is mediated by cytokines (*e.g.*, TNF-α, IL-1β, IL-6), chemokines (*e.g.*, MCP-1, MCP-5, MIP-1β) and cell adhesion molecules [*e.g.*, ICAM-1, platelet-endothelial cell-adhesion molecule-1 (PECAM-1)], which are upregulated immediately after noise exposure[17,46,69-71]. Fujioka *et al*[46] demonstrated an upregulation of the proinflammatory cytokines TNF-α, IL-1β and IL-6 in the noise-damaged cochlea as early as 3 h after noise exposure. IL-6 immunoreactive cells were observed initially in the lower and lateral regions of the spiral ligament, specifically in the cytoplasm of type Ⅳ and Ⅲ fibrocytes, then throughout the spiral ligament and even in the stria vascularis[46]. Double labelling with NeuN, a neuronal marker, showed IL-6 expression in the spiral ganglion neurons 12-24 h after noise exposure. IL-6 upregulation in the noise-exposed cochlea likely contributes to cochlear injury, as the inhibition of IL-6 suppressed cochlear inflammation and mitigated the hearing loss[64]. Chemokines that are chemotactic for macrophages such as MCP-1/CCL2, MCP-5/CCL12, and MIP-1β/CCL4 are upregulated in the noise-exposed cochlea 2 h following acoustic trauma[17]. The early expression of chemokines suggests that resident cochlear cells may be responsible for this upregulation.

ICAM-1/CD54 is a vascular adhesion molecule that plays a critical role in mediating temporary adhesion/immobilisation of leukocytes to vascular endothelial cells in preparation for extravasation. Increased expression of ICAM-1 at the protein level is seen 24 h after noise exposure, reaching a maximum at 2 and 4 d, and returning to basal levels by 14 d[17]. This elevated expression is seen chiefly in the vascular endothelial cells and fibrocytes occupying the root region of the spiral ligament, and less intensely in the region of the spiral ligament adjacent to the cochlear bony capsule. The endosteal cells lining the scala tympani and scala vestibuli and capillaries of the stria vascularis also show increased ICAM-1 immunolabelling. Upregulation of ICAM-1 at the mRNA level is first observed 2 h after noise exposure. The increased ICAM-1 expression in these cells regulates and directs the extravasation and cellular infiltration of inflammatory leukocytes. Results from our recent study on ICAM-1 expression fol-

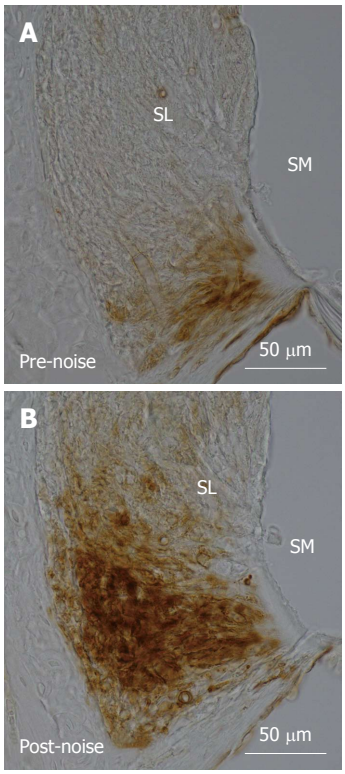

Figure 1 Intercellular adhesion molecule-1 immunolabelling in the spiral ligament of the cochlear basal turn in C57BL/6 mice. A: In the non-noise exposed cochlea, intercellular adhesion molecule-1 (ICAM-1) was expressed by type Ⅳ fibrocytes and vascular endothelial cells in the lowest region of the spiral ligament; B: Mice exposed to traumatic noise (100 dB SPL, 8-16 kHz) for 24 h showed increased expression of ICAM-1, peaking at 24 h following acoustic trauma. ICAM-1 immunolabelling became more intense and expanded to cover a much greater area in the inferior region of the spiral ligament. ICAM-1 immunoexpression was determined by immunoperoxidase histochemistry and photomicrographs of mid-modiolar cochlear sections were taken with a digital light microscope (Nikon Eclipse 80i) at 40 × magnification. SL: Spiral ligament; SM: Scala media.

lowing acute noise exposure in mice are compatible with these findings (Figure 1). Other adhesion molecules that show increased expression following noise exposure include P-selectin, PECAM-1 and VCAM-1[72,73]. Shi *et al*[72] demonstrated that the expression of these adhesion molecules is modulated by poly(ADP-ribose) polymerase-1 (PARP-1), a DNA repair enzyme. They suggested that noise activates PARP-1 in capillary endothelial cells of the spiral ligament and stria vascularis, which may act through NF-κB to regulate the expression of adhesion proteins in the lateral wall.

The expression of many proinflammatory mediators that participate in the acute inflammatory response is broadly regulated by the transcription factor NF-κB. Apart from its pivotal role in immune and inflammatory responses, NF-κB is also implicated in a range of processes such as cell survival, apoptosis, development, differentiation and cell growth[74]. NF-κB comprises a family of five inducible transcription factors, p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), RelB, and c-Rel[75]. They exist as hetero- or homo-dimeric complexes, with the p50/p65 hetero-dimer being the predominant form. In quiescent cells, NF-κB is expressed in the cytoplasm in a latent form, with an inhibitory protein (IκB) bound to the dimer. Upon stimulation, the inhibitory protein is degraded, activating the NF-κB dimer, which then translocates to the nucleus where it binds to the promoters of its target genes. NF-κB activation in the cochlea has been demonstrated following noise exposure[73,76,77]. Following a 2 h exposure of mice to traumatic noise (124 dB SPL), translocation of p65 and p50 to the nucleus of fibrocytes in the lateral wall was observed, indicating NF-κB activation[76]. Prominent nuclear localisation of NF-κB occurred 2 h after noise exposure, but the nuclear immunostaining subsided after 72 h, suggesting an early response of NF-κB to acoustic overstimulation.

As mentioned earlier, a large population of PVMs exist in the stria vascularis, however, these cells are not found elsewhere in the cochlea, including the spiral ligament[52]. The PVMs play an important role in regulating the integrity of the intrastrial fluid-blood barrier by modulating the expression of tight- and adherens-junction proteins between the endothelial cells *via* the secretion of pigment epithelium growth factor (PEDF)[78,79]. The integrity of the barrier is critical for establishing and maintaining the endocochlear potential and preventing the entry of toxic substances into the cochlea[80]. Exposure to excessive noise leads to breakdown and increased permeability of the blood-labyrinth barrier by causing PVMs to change morphology and detach from strial capillaries and also by causing a significant downregulation of PEDF production and tight junction protein expression[81]. Similar to the cochlea, the retina of the eye contains perivascular macrophages, which also contribute to the maintenance of the blood-retinal barrier[82]. Recent evidence has demonstrated that bone marrow-derived cells (BMDCs) are recruited to the stria vascularis during the first week after acoustic injury to repair and restore the noise-damaged blood vessels[83]. These cells promote angiogenesis and neovascularization, differentiating into PVMs, pericytes and endothelial cells and integrating into the strial blood vessels by 4 wk after noise exposure. This recruitment is mediated by an intrinsic (iNOS)-dependent stromal cell-derived factor-1α (SDF-1α) signalling pathway. Blocking the activity of iNOS or SDF-1α significantly reduced both the number of infiltrating BMDCs and the capillary density (vascular repair) in the stria vascularis of the noise-exposed cochlea.

Similar to noise-induced hearing loss, oxidative stress and inflammation are major contributing factors to cisplatin-induced ototoxicity. Cisplatin has been shown to increase the expression of inflammatory mediators such as iNOS, cyclo-oxygenase-2 and TNF-α, which are downstream targets of the transcription factor, signal transducer and activator of transcription-1 (STAT1)[84]. Cisplatin-induced activation of STAT1 is dependent on ROS generation through NOX3, a member of the NOX family of superoxide-generating nicotinamide adenine dinucleotide phosphate oxidases. NOX3 is expressed almost exclusively in the inner ear and serves as the primary

source of ROS generation in the cochlea[85]. siRNA-mediated gene silencing of NOX3 mitigates cisplatin-induced hearing loss, demonstrating a key role of NOX3 in the development of cisplatin-mediated ototoxicity[86]. In contrast to these findings, recent data from our group showed that exposure to noise results in a significant downregulation of NOX3 in the cochlea[87]. We propose that the reduction in NOX3 may represent an endogenous protective mechanism to reduce oxidative stress in the noise-exposed cochlea. These studies provide evidence that NOX3 is involved in the development of noise- and cisplatin-induced cochlear injury, albeit in a different way.

The exact role inflammatory cells play once recruited to the noise-damaged cochlea remains unclear. It is possible that the inflammatory response exacerbates the cellular damage in the cochlea by causing bystander tissue injury. It has also been suggested that the recruitment of inflammatory cells following acoustic injury is part of a wound healing response, given that infiltrating cells are largely observed in the region of the spiral ligament where noise-induced fibrocyte loss is most evident[16,17,49,69]. Leukocytes may play a critical role in promoting repair by removing cellular debris created by the primary insult. These cells may contribute to the repair process by changing the local environment *via* the secretion of chemical mediators such as cytokines and growth factors. Inflammatory leukocytes could function along with resident fibrocytes of the spiral ligament to regulate repair of the noise-damaged cochlear structures. It has been speculated that the fibrocytes initiate the local inflammatory process[65]. These cells express similar cytokines, chemokines and adhesion molecules, and also respond to signals used by leukocytes for cell-cell signalling. Cochlear fibrocytes can perhaps be considered facultative resident macrophages, serving some functions normally performed by circulating macrophages.

TREATMENT STRATEGIES FOR MITIGATING NOISE-INDUCED COCHLEAR INFLAMMATION

At the present time, there is no cure for noise-induced hearing loss, or any other types of hearing loss. The only therapeutic intervention for the hearing impaired is the use of hearing devices such as hearing aids that amplify sound or cochlear implants. A cochlear implant is a neural prosthesis that functions by electrically stimulating residual spiral ganglion neurons, the primary auditory neurons of the cochlea.

Corticosteroids (glucocorticoids) are widely used in the treatment of numerous acute and chronic inflammatory diseases, and have also long been used in the management of SNHL of various causes, including noise-induced hearing loss. Corticosteroids are typically administered systemically, either intravenously or orally. Appropriate doses of steroids supress excessive inflammation, but are unable to completely recover the associated hearing loss. Higher doses, on the other hand, can be deleterious to cochlear function in the long term and are often accompanied by a wide range of adverse side effects[88]. Glucocorticoids exert their actions by binding to and activating soluble cytoplasmic glucocorticoid receptors, which translocate to the nucleus and bind to specific DNA sites, culminating in the downregulation of proinflammatory cytokines and adhesion molecules[89]. Experiments have demonstrated that dexamethasone, a popular glucocorticoid, suppresses TNF-α-induced inflammatory mediator release from cultured spiral ligament fibrocytes[54]. The otoprotective effects of steroids may be mediated through the actions of NF-κB, as glucocorticoids are shown to be potent inhibitors of NF-κB activation *via* the induction of the IκBα inhibitory protein[90]. Local routes of steroid delivery have been developed without the unfavourable side effects. Direct infusion of dexamethasone into the perilymphatic space using osmotic mini-pumps has been reported to show protective effects against noise-induced injury in the guinea pig cochlea[91]. Intratympanic administration of steroids have also shown good therapeutic efficacy[92].

From our existing knowledge of the underlying mechanisms and pathways of the cochlear inflammatory response, rational therapeutic approaches can be devised to supress the inflammation and reduce cochlear injury. It is speculated that there are networks in the cochlea among inflammatory cells, fibrocytes and vascular endothelial cells, which are interconnected by various proinflammatory mediators (chemokines, cytokines, and adhesion molecules)[47]. Appropriate control of these networks could potentially attenuate the inflammatory reaction in the cochlea. Because of their early expression in the inflammatory response and their role in recruiting inflammatory cells into the cochlea, targeting chemokines/cytokines through direct inhibition may represent an effective novel therapeutic strategy.

Satoh *et al*[56] examined the therapeutic potential of anti-TNF-α therapy and showed that blocking the activity of TNF-α using Etanercept, a soluble TNF-α receptor-FC fusion protein, significantly attenuated the cochlear inflammatory response (reduced inflammatory cell infiltration and cochlear fibrosis) in an animal model of immune-mediated labyrinthitis induced by immunisation with KLH. A further study showed that neutralisation of TNF-α using Etanercept markedly decreased the expression and secretion of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in the cochlea after cisplatin injection[21].

Another potential treatment strategy would be to block IL-6 signalling in the cochlea. It is interesting in this regard that specific humanised neutralising antibodies against IL-6 have recently been used clinically with promising effects in patients with rheumatoid arthritis and inflammatory bowel disease. In fact, a recent study by Wakabayashi *et al*[64] showed that inhibition of IL-6 with IL-6 receptor neutralising antibody (MR16-1) resulted in a dramatic suppression of the cochlear inflammatory response (reduced infiltration of inflammatory cells) and significantly improved hearing function in noise-exposed mice.

Recently, Nakamoto *et al*[70] showed that administration of geranylgeranylacetone (GGA), an anti-ulcer drug,

suppressed the expression of proinflammatory cytokines (IL-6 and IL-1β) in the noise-exposed cochlea and also improved auditory function. GGA activates heat shock transcription factor 1 (HSF1), which induces the expression of heat shock proteins. HSF1 is also known to directly or indirectly regulate cytokine expression, such as inhibiting the expression of IL-6 and IL-1β. GGA can also reduce inflammation in other organs (*e.g.*, liver) without apparent side effects even at large doses. GGA may therefore provide a novel beneficial strategy for the prevention of noise-induced hearing loss.

The role of antioxidants in noise-induced hearing loss has been the subject of extensive research. Antioxidants have been demonstrated to provide a protective effect in the cochlea by restoring the redox balance. A recent study examined the effects of antioxidant treatment on the inflammatory response in the cochlea following noise exposure[67]. This study reported that antioxidant treatment not only reduced markers of oxidative stress, but also significantly reduced the infiltration of inflammatory cells into the cochlea. This finding suggests an anti-inflammatory role of antioxidants in the cochlea.

Extensive evidence from *in vitro* and *in vivo* studies has demonstrated the strong anti-inflammatory potential of adenosine, a ubiquitous signalling molecule and neuromodulator, in a range of tissues[93-98]. Adenosine exerts its anti-inflammatory action by influencing almost all aspects of the immune response[99]. The A_{2A} receptor, reported to be the crucial receptor involved in the suppression of inflammation, is a promising target for the treatment of inflammatory conditions. Selective A_{2A} receptor agonists have been used successfully in the therapy of sepsis, inflammatory bowel disease, skin inflammation and arthritis[98], and a similar effect could be postulated in the cochlea. In addition, A_{2A} receptor agonists have been reported to suppress neuroinflammation in animal models[99]. In the mammalian (rat) cochlea, A_{2A} receptors are expressed in the inner hair cells and supporting Deiters' cells of the organ of Corti, spiral ligament, spiral ganglion neurons, and blood vessels[100]. This broad distribution suggests an important role of A_{2A} receptors in the cochlea. The systemic administration of exogenous adenosine is limited by its peripheral side effects[97]. An alternative approach for augmenting the availability and actions of endogenous adenosine that has received increasing attention in recent years is the inhibition of adenosine kinase[94,101,102]. Adenosine kinase inhibitors, such as ABT-702, have demonstrated excellent efficacy in animal models of acute and chronic inflammation[101,103,104], and may have considerable therapeutic potential in cochlear inflammation. Adenosine kinase is extensively distributed in the adult cochlea[105,106], and may have a critical role in the regulation of adenosine signalling under physiological and pathological conditions.

CONCLUSION

The cochlea responds to trauma and infection like organs elsewhere in the body by eliciting an inflammatory response. Exposure to excessive noise triggers a cochlear inflammatory response that is characterised by an initial up-regulation of numerous proinflammatory mediators and adhesion molecules by various resident cochlear cell types, followed by the rapid recruitment and infiltration of inflammatory cells into the cochlea from the systemic circulation. Much has been learned over the years of the noise-induced inflammatory process in the cochlea from animal models, but the exact mechanisms by which noise elicits this response is still unclear. The noise-induced inflammatory response may be involved in propagating cellular damage in the cochlea, but there is also a possibility that it may be involved in reparative processes. The mechanism and importance of this response in the noise-injured cochlea requires further exploration. With deeper knowledge of the underlying cochlear inflammatory response, we can explore and develop novel therapeutic interventions to protect cochlear tissues from inflammation-induced injury and noise-induced hearing loss.

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Adenosine amine congener ameliorates cisplatin-induced hearing loss

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Abstract

AIM: To investigate a novel pharmacological intervention to mitigate cisplatin ototoxicity using a selective adenosine A_1 receptor agonist adenosine amine congener (ADAC).

METHODS: Male Wistar rats (8-10 wk) were exposed to a two-cycle cisplatin treatment similar to clinical course of cancer chemotherapy. Each cycle comprised 4 d of intraperitoneal cisplatin injections (1 mg/kg twice daily) separated by 10 d of rest. ADAC (100 μg/kg) or drug vehicle solution (control) was administered intraperitoneally for 5 d at 24 h intervals during the second cisplatin cycle (Regime 1), or upon completion of the cisplatin treatment (Regime 2). Hearing thresholds were measured using auditory brainstem responses (ABR) before cisplatin administration (baseline) and 7 d after the end of cisplatin treatment. Histological analysis of cochlear tissues included hair cell counting and qualitative assessment of apoptosis using terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) staining.

RESULTS: ABR threshold shifts in cisplatin-treated Wistar rats ranged from 5-29 dB across the frequency range used in the study (4-24 kHz). Higher frequencies (16-24 kHz) were mostly affected by cisplatin ototoxicity (mean threshold shift 25-29 dB). ADAC treatment during the second cisplatin cycle reduced cisplatin-induced threshold shifts by 12-16 dB ($P < 0.01$) at higher frequencies compared to control vehicle-treated rats. However, the treatment was ineffective if ADAC administration was delayed until after the completion of the cisplatin regime. Functional recovery was supported by increased survival of hair cells in the cochlea. Qualitative analysis using TUNEL staining demonstrated reduced apoptosis of the outer hair cells and marginal cells in the stria vascularis in animals treated with ADAC during the second cisplatin cycle.

CONCLUSION: A_1 adenosine receptor agonist ADAC mitigates cisplatin-induced cochlear injury and hearing loss, however its potential interference with antineoplastic effects of cisplatin needs to be established.

Key words: Cisplatin; Cochlea; Ototoxicity; Hearing loss; Adenosine receptors; Adenosine amine congener; Otoprotection

Core tip: This study investigated a novel pharmacological intervention to mitigate cisplatin ototoxicity using

systemic administration of a selective adenosine A1 receptor agonist adenosine amine congener (ADAC). Our study demonstrates that systemic administration of ADAC confers partial protection from cisplatin-induced ototoxicity. In rats exposed to cisplatin, ADAC ameliorated high frequency hearing loss, improved the survival of the outer hair cells and reduced apoptosis of the outer hair cells and marginal cells in the stria vascularis. This study provides support for the otoprotective role of ADAC with potential clinical benefits extending from noise-induced hearing loss to cisplatin ototoxicity.

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INTRODUCTION

Two major classes of therapeutic agents can induce sensorineural hearing loss: aminoglycoside antibiotics and platinum-containing chemotherapy agents[1]. These drugs primarily target the outer hair cells in the basal region of the cochlea and cause high frequency sensorineural hearing loss. Oxidative stress, triggering downstream cell death signalling pathways, appears to be the common mechanism of ototoxicity[1,2].

Platinum-containing agents, such as cisplatin, carboplatin and oxaliplatin, are widely used to treat malignancies ranging from testicular, ovarian and bladder cancers to lung, head and neck malignancies[3]. These platinum complexes cause cross-linking of DNA and proteins and formation of adducts which ultimately trigger apoptosis in tumor cells[1,2]. The use of these anti-cancer drugs is limited by serious side effects, which include nephrotoxicity, neurotoxicity, gastrointestinal toxicity, leukopenia, thrombocytopenia and ototoxicity[4]. Cisplatin is considered the most ototoxic among platinum-containing agents, but it is generally more effective than carboplatin and oxaliplatin against different forms of cancer[4]. Cisplatin ototoxicity affects most patients, and is mainly manifested as tinnitus and bilateral high-frequency hearing loss, in the absence of the vestibular symptoms[4]. With prolonged cisplatin treatment, hearing loss extends to lower frequencies necessary for speech perception, and the spiral ganglion neurons degenerate concomitantly with the loss of hair cells[5]. Platinated DNA has been detected in the nuclei of the outer hair cells, marginal cells of the stria vascularis and the fibrocytes of the spiral ligament[6]. Cross-linking of DNA by cisplatin may lead to p53-mediated apoptosis of the outer hair cells and the lateral wall tissues, the spiral ligament and stria vascularis[7-9]. A reduction in the endocochlear potential, probably resulting from a dysfunctional stria vascularis, often precedes outer hair cell loss in the acute model of cisplatin ototoxicity[10].

Cisplatin reacts with the outer hair cells to form the highly reactive monohydrate complexes[1], and these complexes activate the NOX3 isoform of NADPH oxidase, which in turn generates superoxide[11-13]. This leads to formation of more toxic reactive oxygen species (ROS), such as hydroxyl radicals and peroxynitrite[2]. Excessive ROS production can overwhelm endogenous anti-oxidant mechanisms (*e.g.*, glutathione, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase), and activate c-Jun *N*-terminal kinase signalling pathways in cochlear tissues, leading to apoptosis *via* caspase-dependent mechanisms[1]. ROS can also activate nuclear factor κB (NF-κB), which regulates the expression of pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α[14] and triggers the inflammatory cascade.

Cisplatin ototoxicity can be ameliorated by various protective agents targeting oxidative stress, inflammation and apoptosis[15]. Enhancing the endogenous antioxidant system of the cochlea and administration of free radical scavengers has been the main approach to reduce ototoxic effects of cisplatin[2,16]. Systemic antioxidant administration, however, can interfere with the anti-tumour activity of cisplatin, whilst local (intratympanic) administration is an invasive procedure with its own limitations[2,16]. There is no ideal protective agent for clinical use at present, and finding safe and effective treatments for cisplatin ototoxicity would significantly improve the quality of life of many cancer sufferers. In this study, we investigated a novel pharmacological intervention to mitigate cisplatin ototoxicity using systemic administration of a selective adenosine A_1 receptor agonist adenosine amine congener (ADAC).

It has been shown that cisplatin treatment induces a fivefold increase in adenosine A_1 receptor expression in the chinchilla cochlea[17], suggesting a potential role of these receptors in cochlear response to cisplatin. Other studies have reported that the local administration of adenosine A_1 receptor agonists *R*-phenylisopropyladenosine (*R*-PIA) or 2-chloro-*N*6-cyclopentyladenosine (CCPA) to the round window membrane of the cochlea reduces cisplatin-induced auditory threshold shifts[18]. The capacity of A_1 receptor agonists to protect the cochlea from cisplatin opened a new realm of therapeutic strategies to combat cisplatin ototoxicity.

Systemic administration of A_1 receptor agonists is generally limited by their cardiovascular side effects[19]. However, a selective A_1 receptor agonist ADAC is characterized by reduced cardiovascular side effects (bradycardia, hypotension and hypothermia) compared to other drugs acting on adenosine A_1 receptors[20,21]. This suggests that ADAC can be administered systemically, avoiding the surgical procedures required to deliver drugs to the inner ear. Our previous studies have shown that ADAC can attenuate noise-induced hearing loss and ameliorate cochlear injury in instances of acute and extended noise exposure[22]. The improvement of hearing thresholds was supported by increased survival of sensory hair cells and reduced

expression of oxidative stress markers in the cochlea. Here, we demonstrate that ADAC ameliorates cochlear injury and partially prevents cisplatin-induced hearing loss, providing further support for the role of A_1 receptors in cochlear protection from ototoxic anti-cancer drugs.

MATERIALS AND METHODS

Animals

Male Wistar rats (8-10 wk) were used in this study. Animals with pre-existing hearing loss or abnormalities in the external or middle ear were excluded from the study. All procedures complied with international guidelines for the ethical use of animals and were approved by the University of Auckland Animal Ethics Committee.

Cisplatin administration

A two-cycle cisplatin treatment resembling a clinical course of chemotherapy was used in this study. This model has a low mortality rate, and provides an excellent tool to study cisplatin ototoxicity and its prevention[23]. Each cycle consists of 4 d of cisplatin injections (1 mg/kg *ip* twice daily) separated by 10 d of rest. Each rat received a total of 16 mg/kg of cisplatin. Cisplatin (Sigma Aldrich) was dissolved in saline (0.5 mg/mL), aliquoted and stored at -20 ℃. Cisplatin aliquots were heated in a 37 ℃ water bath before administration.

Treatment with ADAC

ADAC treatment was initiated concomitantly with the second cycle of cisplatin administration (Regime 1) or immediately after completion of cisplatin administration (Regime 2) (Figure 1). In both studies, ADAC was given as five daily injections (100 μg/kg per day *ip*) at 24 h intervals. This is the same dosing schedule that was previously used to mitigate noise-induced hearing loss[22]. In the control group, injections of the drug vehicle (200 μL/100 g per day *ip*) were administered at the same intervals as ADAC. ADAC (Sigma-Aldrich) was dissolved in 1 mol/L HCl and then in 0.1 mol/L phosphate buffered saline (PBS; pH 7.4) to prepare a 50 μg/mL stock solution, as described previously[22]. The stock solution was then aliquoted and stored at -20 ℃. Light-protected ADAC aliquots were heated in a 37 ℃ water bath for 30 min before administration.

Auditory brainstem responses

Auditory brainstem responses (ABR) were measured before cisplatin administration (baseline) and 7 d after the end of cisplatin treatment. The acoustic stimuli for ABR were produced and the responses recorded using a digital signal processing package and associated BioSig software (Tucker Davis Technologies, Alachua, FL, United States). ABR measurements were conducted in a double walled sound attenuating chamber (Shelburg Acoustics, Pty Ltd., Croydon North, Australia). The rats were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg), and placed on a heating pad to maintain body temperature at

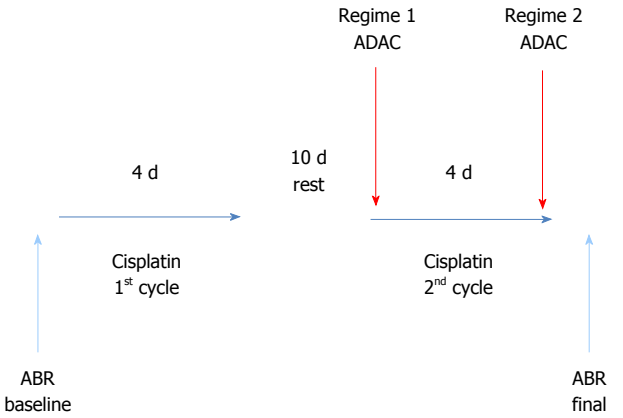

Figure 1 Study design. Cisplatin injections (1 mg/kg *ip*) were given twice daily in two cycles separated by 10 d of rest, and adenosine amine congener (ADAC) (100 μg/kg *ip*) was administered as five daily injections at 24 h intervals. ADAC treatment was administered along with the second cisplatin cycle (Regime 1) or immediately after completion of both cycles (Regime 2).

37 ℃. To obtain ABR responses, fine Grass F-E3 stainless steel electrodes were placed subdermally at the vertex (reference), at the mastoid region of the ear of interest (active electrode) and the ground electrode was inserted at the mastoid region of the opposite ear. The electrodes were attached to a TDT Bioamp head stage and amplified 100000 times in a TDT DB4 amplifier. Sound stimuli were supplied *via* a DT 48 Beyerdynamic transducer connected to a 10 cm plastic tubing placed into the external auditory canal of the animal's ear. Rats were tested by applying a series of tone pips (5 ms duration, 1.5 ms rise and fall times) at varying intensities to determine the auditory threshold at the set frequency (4-24 kHz). The threshold of the ABR complex (waves i-v) were determined by progressively attenuating the sound intensity in 5 dB steps until the wave i-v complex of the averaged ABR waveforms was no longer distinguishable from noise floor in recorded traces. The ABR threshold was defined as the lowest intensity (to the nearest 5 dB) at which a response could be visually detected above the noise floor. Repeat waveforms were analysed at each frequency to determine the consistency of the responses and to identify the recurring peaks.

Hair cell counts

After the last ABR measurement, rats from the first ADAC treatment group (Regime 1, see Figure 1) were euthanised with an overdose of anaesthetic (Pentobarbitone, 100 mg/kg *ip*) and cochleae removed for histological analysis. After the overnight fixation in 4% paraformaldehyde (PFA), the cochleae were decapsulated and the organ of Corti removed. The surface preparation of the organ of Corti was separated into the apical, middle and basal turns, and the tissues were permeabilized with 1% Triton-X 100 for 1 h. Alexa Fluor 488 phalloidin (Invitrogen) dissolved in 0.1 mol/L PBS (pH 7.4) was used to stain F-actin in the hair cells and their stereocilia. Tissues were incubated in 1% phalloidin (2 U/mL) for 40 min,

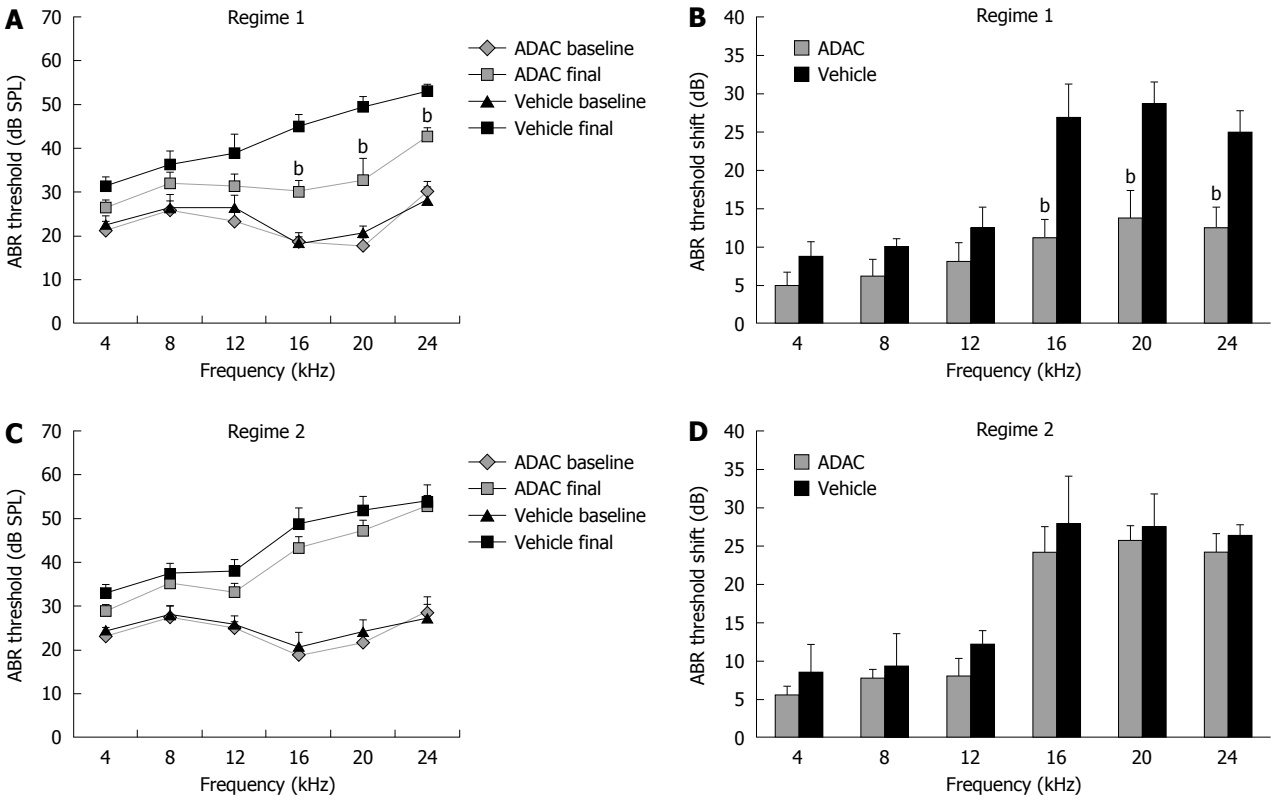

Figure 2 The effect of adenosine amine congener on cisplatin-induced auditory brainstem responses threshold shifts. A: Auditory brainstem responses (ABR) thresholds before (baseline) and 7 d after cisplatin administration (final). Adenosine amine congener (ADAC) was co-applied with cisplatin during the second cycle (Regime 1); B: ADAC reduced ABR threshold shifts when administered concomitantly with the second cisplatin cycle; C: ABR thresholds before (baseline) and 7 d after cisplatin administration (final). ADAC was administered after the completion of cisplatin treatment (Regime 2); D: ADAC had no effect on cisplatin-induced threshold shifts when applied after the completion of cisplatin treatment. In the control group, injections of the vehicle solution were administered at the same intervals as ADAC. ABRs were measured in response to tone pips (4-24 kHz). Data are expressed as mean ± SE (n = 8). $^{b}P < 0.01$ *vs* control group, one-way analysis of variance.

washed with PBS for 30 min, and mounted onto glass slides using Citifluor AF1 antifading mounting medium (Agar Scientific, London, United Kingdom). The slides were visualised using a Zeiss epifluorescence microscope equipped with an Axiocam camera and Axiovision v3.1 software. Images were taken for the entire length of the cochlea, and the number of missing hair cells was counted for each turn and presented as a percentage of total number of hair cells in that turn.

Assessment of cell death using transferase mediated dUTP nick end labelling staining

Apoptosis in the cisplatin-treated rat cochleae was identified by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labelling (TUNEL) using a commercial *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). After fixation with 4% PFA, decalcification in 5% EDTA solution for 7 d and overnight cryoprotection in 30% sucrose, the cochleae were embedded in Tissue-Tek optimal cutting temperature compound (OCT, Miles Laboratories, Elkhart, IN, United States), snap-frozen in isopentane, and stored at -80 ℃. Mid-modiolar cochlear cryosections (30 μm) from ADAC- and vehicle-treated rats were permeabilised with 1% Triton X-100 and blocked with 5% normal goat serum in PBS for 1 h. The sections were washed and incubated with the TUNEL reaction mixture (fluorescein nucleotide label solution and TdT enzyme solution at 1:10 dilution) for 2 h at 37 ℃ in a dark humidified chamber. Negative controls were incubated with the label solution only. The sections were rinsed several times in PBS, mounted in Citifluor, and visualised using a laser scanning confocal microscope (FluoView™ FV1000, Olympus) and processed with Olympus FluoView v.1.6a software. The cochleae obtained from the ADAC treatment Regime 1 were analysed and the images representative of at least five individual experiments are shown.

Statistical analysis

Results were presented as the mean ± SE (n = 8 per group). The comparison of ABR thresholds was performed using one-way analysis of variance followed by a Holm-Sidak pairwise multiple comparison and hair cell loss was analysed using a Student's unpaired t-test assuming unequal variances. The α level was set at 0.05.

RESULTS

ABR threshold shifts

ABR were used in this study as a standard functional method of assessing cochlear function in animals. ABR thresholds were measured prior to the start of the first

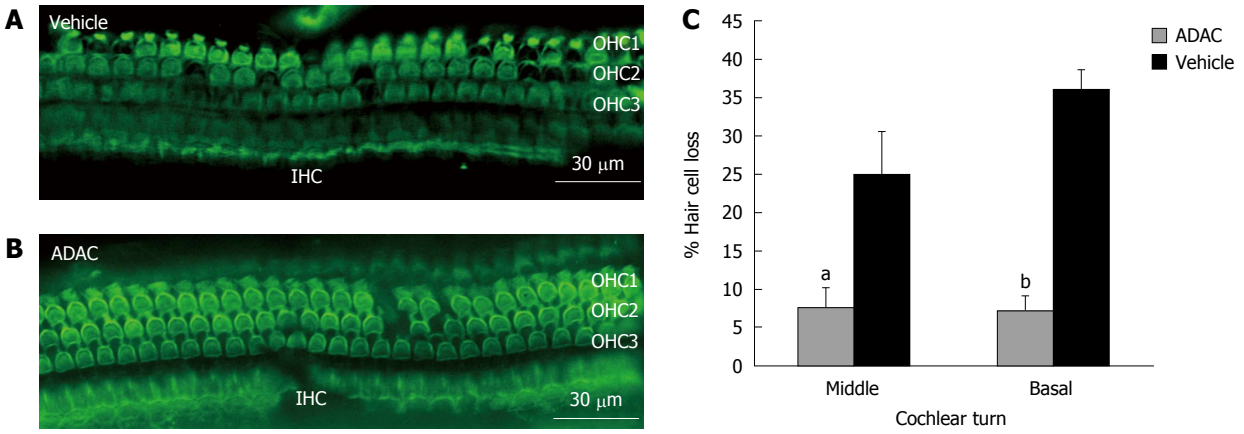

Figure 3 The effect of adenosine amine congener on hair cell loss in the rat cochleae exposed to cisplatin (Regime 1). A: The surface preparation of the middle turn organ of Corti in the vehicle-treated cochlea; B: The middle turn organ of Corti in the adenosine amine congener (ADAC)-treated cochlea; C: Percentage of hair cell loss in the cochleae exposed to cisplatin treated with ADAC or drug vehicle solution. Data presented as mean ± SE (n = 8). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control grou, unpaired t test. IHC: Inner hair cells; OHC: Outer hair cells.

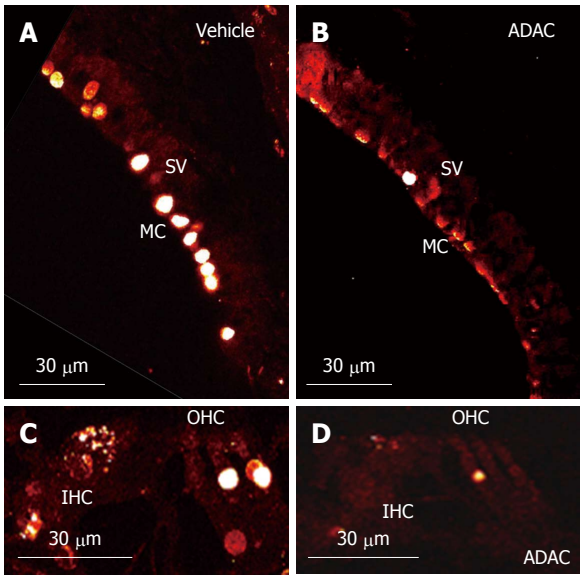

Figure 4 Transferase mediated dUTP nick end labelling staining in the rat cochleae exposed to cisplatin. A: Apoptotic marginal cells (MC) of the stria vascularis (SV) in the control vehicle-treated cochlea; B: Reduced number of apoptotic marginal cells in the adenosine amine congener (ADAC)-treated cochlea; C: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay staining in the organ of Corti of the control vehicle-treated cochlea; D: Reduced apoptosis in the organ of Corti of the ADAC-treated cochlea. Images are single optical sections of the middle turn. IHC: Inner hair cells; OHC: Outer hair cells.

cisplatin cycle and 7 d after the completion of cisplatin treatment, and the threshold shift was calculated for each animal as a difference between these two measurements. Baseline ABR thresholds before cisplatin administration were similar in all groups of animals (Figure 2A and C). Intraperitoneal administration of cisplatin caused significant elevation of ABR thresholds in all animals (Figure 2), which was more substantial at higher frequencies (16-24 kHz; mean threshold shift 25-29 dB). ADAC treatment during the second cycle of cisplatin (Regime 1) reduced ABR threshold shifts by 12-16 dB at higher frequencies ($P < 0.01$) compared to control vehicle-treated rats (Figure 2B). However, when ADAC treatment was delayed until after the completion of both cisplatin cycles (Regime 2), there was no improvement of ABR thresholds (Figure 2D).

Hair cell loss

To determine the extent of hair cell loss with ADAC treatment during the second cycle of cisplatin administration (Regime 1), the outer hair cells were counted in the basal, middle and apical turns of the cochlea. Figure 3A is a representative image of the surface preparation of the middle cochlear turn showing cisplatin-induced loss of the outer hair cells in the vehicle-treated cochlea. The inner hair cells were mostly unaffected by exposure to cisplatin. The survival of the outer hair cells was improved in the ADAC-treated cochlea (Figure 3B), suggesting a cytoprotective effect of this compound. Quantitative assessment of the cisplatin-induced hair cell loss in the vehicle-treated cochleae (Figure 3C) demonstrated a high percentage of missing hair cells in the basal and middle turns (36% and 25%, respectively), whilst the hair cell loss in the apical turn was less than 5% (data not shown). ADAC treatment during the second cisplatin cycle significantly ($P < 0.05$ for the middle turn and $P < 0.001$ for the basal turn) reduced hair cell loss in the basal and middle turns of the cochlea (Figure 3C).

TUNEL staining

TUNEL staining is a technique used for detection of apoptosis at a single cell level, based on labeling of fragmented DNA in the nuclei of apoptotic cells. In this study, TUNEL staining was used for qualitative assessment of apoptosis in the midmodiolar cross-sections of the cisplatin-treated cochlea (Figure 4). This study was performed on cochlear tissues of animals treated with ADAC or vehicle solution during the second cycle of cisplatin treatment (Regime 1). TUNEL staining was mostly limited to the marginal cells of the stria vascularis and the

outer hair cells, whilst the inner hair cells were occasionally positive for TUNEL staining (Figure 4A and C). As expected, the number of apoptotic cells was the highest in the basal turn, slightly lower in the middle turn and minimal in the apical turn. In the basal and middle turns, we mostly observed 1 out of 3 or 2 out of 3 TUNEL-positive outer hair cells, whilst in the apical turn TUNEL-positive cells were observed only occasionally. Supporting cells were mostly unaffected except in the basal turn, where some TUNEL-positive Deiters' cells were observed. Figure 4C shows two TUNEL-positive outer hair cells and an inner hair cell in an advanced stage of apoptosis, judged by diffuse TUNEL staining which correlates with disrupted chromatin and cellular disintegration. In all turns, however, there were more advanced stage outer hair cells than inner hair cells, and in extreme cases complete disintegration of the outer hair cells was observed. TUNEL staining in the spiral ganglion was limited to satellite cells, whilst the neurons appeared unstained (data not shown). ADAC treatment during the second cycle of cisplatin regime consistently decreased TUNEL staining in the stria vascularis and the organ of Corti in the basal and middle turns of the cochlea (Figure 4B and D).

DISCUSSION

Our study demonstrates that systemic administration of ADAC, a selective A_1 adenosine receptor agonist, confers partial protection from cisplatin-induced ototoxicity. In rats exposed to cisplatin, ADAC ameliorated high frequency hearing loss and improved the survival of the outer hair cells. As a qualitative outcome, ADAC treatment reduced apoptosis of the outer hair cells and marginal cells in the stria vascularis.

In this study we have taken advantage of the two-cycle model of cisplatin treatment, which mimics the clinical course of chemotherapy[23] and avoids a high mortality rate known to exist in other animal models of cisplatin toxicity[24,25]. A previous study using the two-cycle model[23] established that hearing loss and the loss of outer hair cells occurs after the second cycle of cisplatin injections, suggesting that this period should be therapeutically targeted to reduce cisplatin ototoxicity. Indeed, ADAC was most effective when administered concomitantly with cisplatin during the second cycle. After the completion of cisplatin treatment, the ototoxic effects of cisplatin were irreversible and the hearing loss was permanent. These results suggest that the role of ADAC is to facilitate the recovery process of the hair cells and other cochlear tissues at early stages of cochlear injury.

Previous studies have shown that apoptotic cell death is the main mechanism of cisplatin ototoxicity[2,4]. In the present study, ADAC reduced the loss of outer hair cells and apoptosis in the organ of Corti and stria vascularis, cochlear tissues particularly vulnerable to cisplatin ototoxicity[2,26]. Our results thus suggest that ADAC improves the survival of sensory and secretory tissues critical for normal cochlear functioning.

It is possible that ADAC targets multiple mechanisms of cochlear injury, reducing oxidative stress and apoptosis upon stimulation of adenosine A_1 receptors. It has been established that A_1 receptors exert a strong cytoprotective role in the cochlea[18,22,27-29], most likely by inducing the activation of antioxidant enzymes[30,31]. A_1 adenosine receptors are up-regulated after local cisplatin administration to the cochlea, and this is considered to be a compensatory mechanism to counter excessive ROS production[17]. Local administration of *R*-PIA (A_1 adenosine receptor agonist) to the round window membrane can increase the production of antioxidant enzymes superoxide dismutase and glutathione peroxidase and significantly reduce the levels of malondialdehyde, a marker of lipid peroxidation[32]. In addition, selective A_1 adenosine receptor agonists, such as CCPA and ADAC, reduce the production of a toxic metabolite nitrotyrosine (marker of oxidative stress) in the noise-exposed cochlea[22,29], providing further support for antioxidant actions of adenosine A_1 receptor agonists. Anti-apoptotic activity of A_1 receptors has also been established. A_1 receptors are positively coupled to extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases[33], known to mediate pro-survival signals in tissues[34]. In addition, the increased expression of adenosine receptors in response to oxidative stress is regulated by transcription factors NF-κB and activator protein 1, which facilitate cell survival in tissues exposed to oxidative stress[35].

A_1 adenosine receptors have been previously shown to reduce cisplatin-induced auditory threshold shifts after local administration of adenosine A_1 receptor agonists, such as *R*-PIA or CCPA, onto the round window membrane of the cochlea[18]. The potential advantage of ADAC in comparison with other adenosine A_1 receptor agonists is the possibility of systemic administration due to reduced cardiovascular effects[20-22]. However, translation from animal models to clinical practice is essential to assess the effectiveness of ADAC. Further studies are required to assess potential side effects, optimal dose and route of drug administration, and bioavailability. Another important caveat is to establish whether systemic administration of ADAC interferes with anti-cancer effects of cisplatin. Nevertheless, this study provides further support for the otoprotective role of ADAC with potential clinical benefits extending from noise-induced hearing loss to cisplatin ototoxicity.

COMMENTS

Background

Cisplatin is one of the most commonly used chemotherapeutic agents highly effective in treatment of various malignancies. The principal dose-limiting side effects of cisplatin include ototoxicity, neurotoxicity and nephrotoxicity. Cisplatin ototoxicity affects most patients, and is manifested as tinnitus and bilateral high-frequency hearing loss.

Research frontiers

Cisplatin ototoxicity can be ameliorated by various protective agents targeting oxidative stress, inflammation and apoptosis. There is no ideal protective agent for clinical use at present, and finding safe and effective treatments for cisplatin ototoxicity would significantly improve the quality of life of many cancer sufferers.

Innovations and breakthroughs

Cisplatin-induced cochlear injury and hearing loss can be reduced after local administration of adenosine A_1 receptor agonists. The advantage of adenosine amine congener (ADAC) in comparison with other adenosine A_1 receptor agonists is the possibility of systemic (*e.g.*, oral) administration due to reduced cardiovascular effects of ADAC at the therapeutic dose.

Applications

This study may lead to therapeutic management of ototoxic side effects in patients receiving cisplatin anti-cancer therapy. An important caveat is to establish whether systemic administration of ADAC interferes with anti-cancer effects of cisplatin.

Terminology

ADAC is a selective A_1 adenosine receptor agonist. Adenosine acts as a cytoprotective substance released from tissues in response to stress. Released adenosine may account for tissue protection and regeneration in a range of tissues *via* adenosine A_1 receptors.

Peer review

The authors present compelling evidence for the ability of ADAC to reduce loss of hearing function and loss of cochlear hair cells induced by cisplatin in Wistar rats, a recognized model of cisplatin ototoxicity. These data are of particular interest due to the possibility of systemically treating humans undergoing cisplatin cancer therapy with ADAC, which has reduced cardiovascular side effects compared to other adenosine agonists.

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CASE REPORT

Extended pectoralis major myocutaneous flap in head and neck reconstruction

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Abstract

Although the pectoralis major myocutaneous flap is often used in head and neck reconstruction, the extension of the skin paddle beyond the inferior limits of the muscle has not been well described. We aim to clarify the design and application of this extended flap in head and neck reconstruction. In this retrospective study, consecutive cases of extended pectoralis major myocutaneous flap reconstruction of post-ablative head and neck defects at a single tertiary referral center were included for analysis. In 7 cases an extended pectoralis major flap was utilized, in which the skin paddle was extended beyond the inferior border of the pectoralis major to include the rectus sheath. Skin and soft tissue as well as composite defects of the oral cavity, parotid/temporal region and neck were reconstructed. All flaps healed satisfactorily with no loss of skin viability. The extended pectoralis major myocutaneous flap is robust and has versatile applications for reconstruction of large, high and three dimensionally complex defects in the head and neck region.

Key words: Head and neck cancer; Surgical flaps; Pedicle flap; Pectoralis major; Extended flap

Core tip: The current report describes the indications, design and technique of the extended pectoralis major flap in reconstructing challenging defects in the head and neck region. The flap has been shown to be safe and robust, and offers an important reconstructive option.

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INTRODUCTION

The pectoralis major flap was first described in 1968[1] and later popularized for head and neck reconstruction by Ariyan[2] in 1979. Due to its ready availability, ease of harvest and reliability, it soon became the choice of reconstruction for post-ablative defects in the head and neck region. Currently however, free tissue transfers, which offer superior pliability and ability to be contoured to the defect, have largely superseded the pectoralis major flap. Nevertheless, the latter retains an important place in contemporary head and neck reconstruction, particularly in resource constrained settings, high risk patients and as salvage after free flap failure.

The principal blood supply to the flap is from the thoracoacromial artery, a branch of the axillary artery that enters the deep muscle surface from beneath the

middle third of the clavicle. In most large series, the skin paddle has been limited to within the surface area of the pectoralis major muscle, *i.e.,* territory supplied by the thoracoacromial artery, as extension beyond this border is thought to compromise blood supply[2,3]. In this report, we describe our experience with the extended pectoralis major flap, wherein the skin paddle was extended beyond the inferior border of the pectoralis major to include rectus sheath.

CASE REPORT

All cases of head and neck defects reconstructed by the pectoralis major myocutaneous flap at our tertiary referral center from 2010 to 2013 were retrospectively reviewed. Among these, cases of extended pectoralis major flap reconstructions were selected for analysis. For the purpose of this report, an extended flap was defined as the design and harvest of a skin paddle with the proximal portion overlying the pectoralis major muscle, and the distal portion extending beyond the inferior border of the muscle to include rectus sheath. The dimensions of the skin paddle were as closely matched as possible to that of the defect. The distal margin of the skin paddle was designed in a curvilinear manner to avoid a sharp tip. The pedicle length was designed so as to ensure an adequate arc of rotation (pivoted on the middle third of the clavicle) and sufficient tension free reach of the inferior margin of the skin paddle to the highest or most distal reach of the defect. A curvilinear line was drawn from the supero-lateral corner of the skin paddle to the anterior axillary fold (Figure 1). From this line an inferior flap was raised superficial to the pectoralis fascia to define the inferior border of pectoralis major muscle. This border was assessed in relation to the inferior border of the skin paddle. The portion of skin paddle extending beyond the inferior border of muscle was harvested with the corresponding underlying rectus sheath in a plane just superficial to rectus muscle. The cut margin of rectus sheath was sutured to the subcutaneous layer of the skin paddle to prevent shearing and disruption in blood supply during harvest. Further proximal harvest continued in a plane deep to pectoralis major muscle and superficial to pectoralis minor muscle. The vascular pedicle was identified on the deep surface of pectoralis major and protected during further harvest. If a second lateral pedicle (lateral thoracic artery) was present, it was divided to obtain adequate arc of rotation. Muscle around the vascular pedicle was thinned if necessary to facilitate distal reach of skin paddle. Pectoralis major muscle fibers were released from rib attachments and the humeral head was also detached completely. The flap was finally mobilized superiorly under the neck skin and the skin paddle was sutured to the defect margins in a single layer. Care was taken to ensure minimal tension and kinking of the vascular pedicle.

In the postoperative period, normal saline was infused at the rate of 100-120 cc/h for the first 24 h. Urinary output was monitored with the Foley catheter *in-situ* to ensure it remained above 50 cc/h. Packed red blood cell was transfused to maintain blood hemoglobin concentration at or above 9 g/dL. Oral or nasogastric tube feeding was commenced 24-48 h following surgery and gradually increased to approximately 3 L/d at which point intravenous fluids was completely stopped. The patient's head was kept elevated by 45 degrees and maintained in a neutral position as far as possible. On the first postoperative day, the patient was made to sit in a chair and daily chest physiotherapy was commenced. Ambulation was started on the second postoperative day, and the Foley catheter was typically removed on the third day. In cases that required mucosal repair, nasogastric feeding and nil by mouth orders were continued until at least the 14th postoperative day and full healing of mucosal incision lines. Flap viability was checked on the first postoperative day by needle prick and then by visual inspection of skin color and turgor on a daily basis until discharge from hospital. Any loss of viability, such as skin necrosis, was recorded.

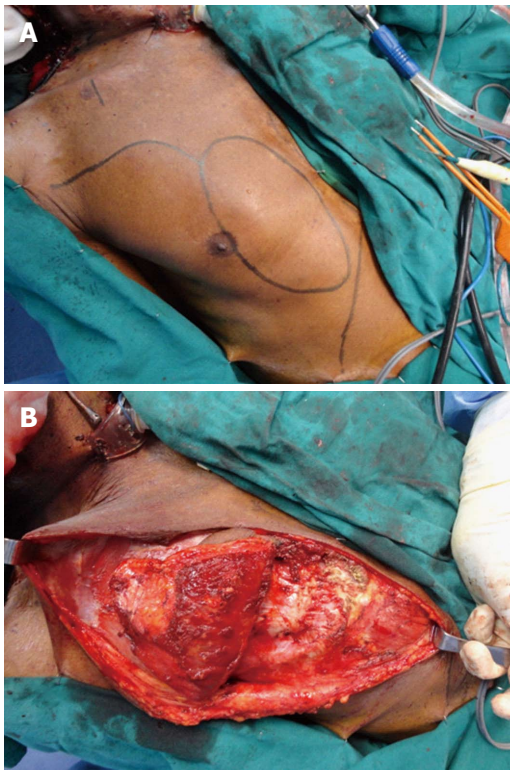

Figure 1 Flap design and elevation. A: Marking of skin paddle with inferior line representing costal margin; B: Flap being elevated along with rectus sheath (exposed rectus muscle inferiorly).

A total of 7 cases of extended pectoralis major myocutaneous flap repairs were done. Patient demographics are given in Table 1. No patient had received preoperative radiotherapy. The distal tip of the skin paddle extended beyond the lower border of pectoralis major by 2-5 cm. In all cases, this cut distal margin was confirmed to exhibit brisk bleeding during harvest. The indications for the extended flap were large defects of mucosal and/or skin surface, high defects involving soft palate or extend-

Table 1 Patient demographics and operative details

Pt No.	Age (yr)/sex	Primary tumor	Defect nature	Approximate skin paddle dimension (cm)
1	60/F	CA thyroid	Large cutaneous and soft tissue defect	7 × 4
2	54/M	CA retromolar trigone	Composite defect of oral mucosa and mandible	6 × 3
3	45/F	CA buccal mucosa	Large full thickness composite defect cheek	5 × 4
4	56/F	CA alveolus	Composite defect of oral mucosa and mandible	6 × 4
5	66/M	CA retromolar trigone	Composite defect of oral mucosa and mandible	6 × 4
6	55/M	CA parotid	Large cutaneous defect of cheek reaching temporal line	8 × 6
7	50/F	CA alveolus	Large full thickness composite defect cheek	8 × 4

F: Femal; M: Male.

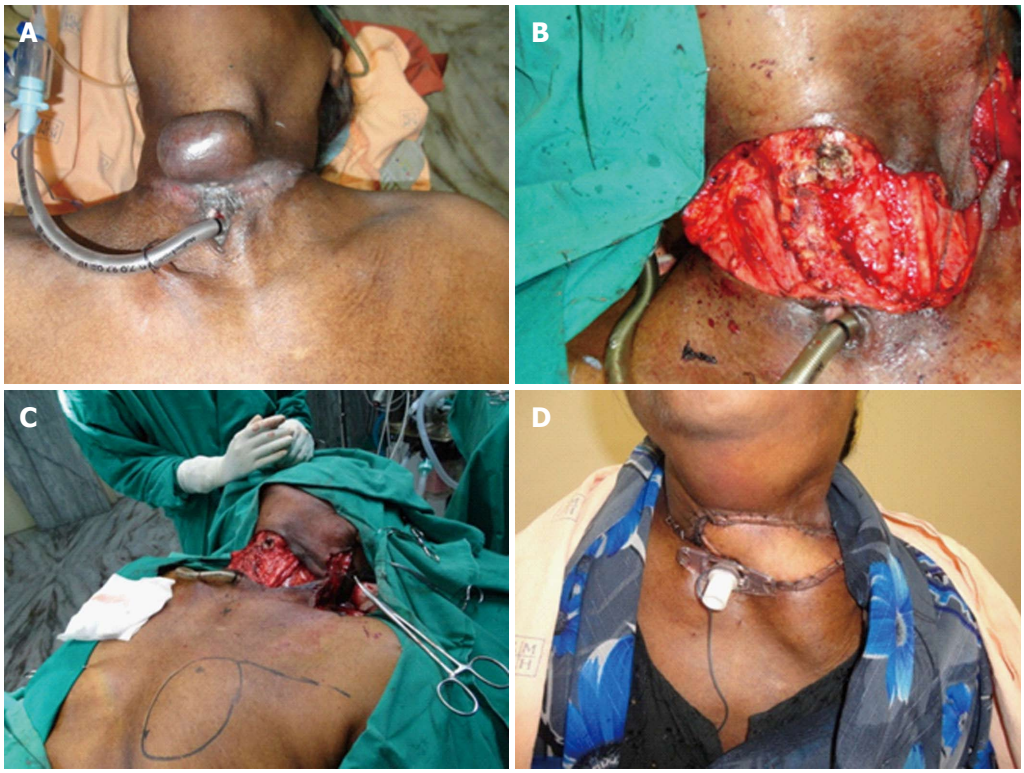

Figure 2 Reconstruction of large cutaneous defect (Patient 1). A: Large locally invasive thyroid malignancy with skin involvement; B: Post-ablative defect; C: Marking for flap; D: Wound 1 wk after surgery.

ing up to and beyond the temporal line, and complex, multi-dimensional intraoral defects requiring tension-free circumferential closure. Patients 3 and 7 had full thickness defects of the cheek with loss of skin, buccal soft tissue and mucosa. In the former patient, the surface area of the cutaneous defect was near-identical to that of the buccal defect (5 cm × 4 cm), while the cutaneous defect was much smaller (2 cm × 1 cm) in the latter. The skin paddle of the flap was used to resurface the cutaneous defect in the former and the buccal defect in the latter. The corresponding large buccal and small external cutaneous defects were reconstructed with split skin grafts. The donor site was closed primarily in 6 cases and a local rotation flap was required in 1 case. No patient required a second simultaneous flap for reconstruction (Figures 2-5).

One patient developed a seroma in the neck anterior to the flap muscle that settled on repeated aspiration. Two other patients developed orocutaneous fistula that healed by daily dressing. All flaps survived fully with no loss of skin viability or necrosis over a minimum follow-up of 60 d. Similarly, there was no major donor site complication.

DISCUSSION

The pectoralis major myocutaneous flap offers a very important reconstructive option in contemporary head and neck surgery. However, limiting the skin paddle to within the surface area of the pectoralis major muscle may occasionally restrict the ability to reconstruct large, high or complex defects. In this report, extension of the skin paddle inferiorly beyond the pectoralis major muscle has been shown not to compromise blood supply and this extended and robust skin paddle can be utilized to reliably reconstruct large or high defects.

The main blood supply to the pectoralis major is the

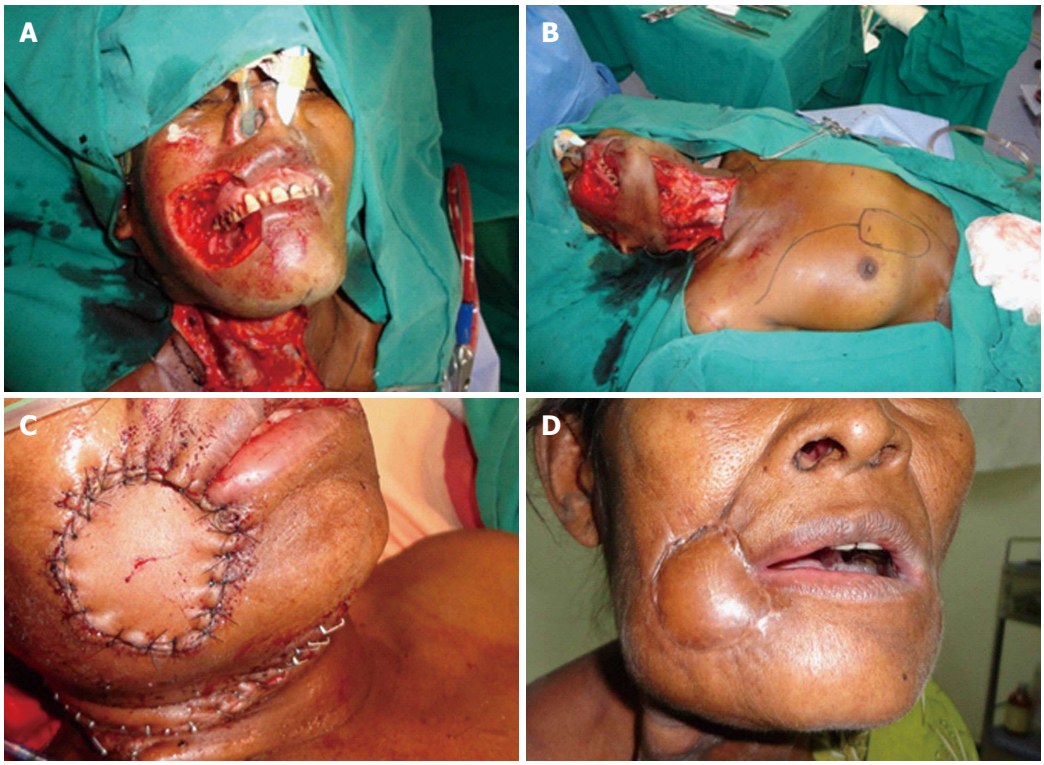

Figure 3 Reconstruction of full thickness cheek defect (Patient 3). A: Post-ablative defect; B: Marking for skin paddle; C: Immediate post-reconstruction; D: Two months following surgery.

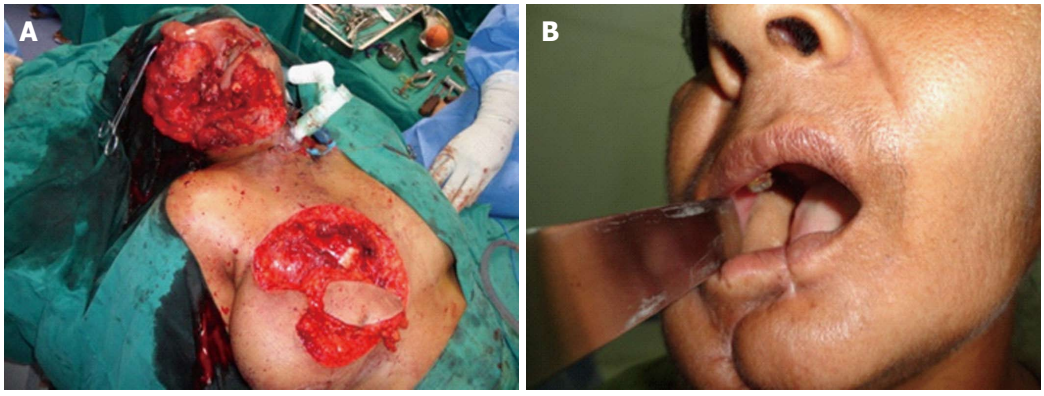

Figure 4 Reconstruction of large mucosal defect (Patient 4). A: Elevation of flap; B: Two months following surgery.

thoracoacromial artery. There are two other vessels supplying the muscle: internal mammary artery with its perforating branches and lateral thoracic artery. The internal mammary vessels continue into the rectus sheath as the superior epigastric artery and vein with large perforators in the periumbilical region of the abdomen. The cutaneous vascular territories of these three vascular systems overlap to supply the skin of the anterior chest and upper abdomen[4]. Cadaveric dye injection studies have confirmed overlap of the skin territories of perforators from the internal mammary, superior epigastric and thoracoacromial systems over the sternum and upper abdominal wall[4,5]. It has been shown that in some cases, the skin paddle of the traditional pectoralis major flap can have a limited supply by the thoracoacromial artery, with the remaining area borne by the perforating branches of the internal mammary artery[6,7]. Extending the skin paddle inferiorly to include the rectus sheath as done in this report can capture the rich fascial vascular plexus of the lower chest and anterior abdominal wall. The distal skin must be designed in a curvilinear fashion to avoid a sharp tip and include the fascia covering the anterior abdominal wall to maintain the fascial vascular plexus. Proximally, the skin island must be designed to overlie the pectoralis muscle to allow the thoracoacromial perforating vessels access into the distal fascial plexus. The edges of the rectus sheath and superficial layers of pectoralis muscle must be sutured to the corresponding subcutaneous layer

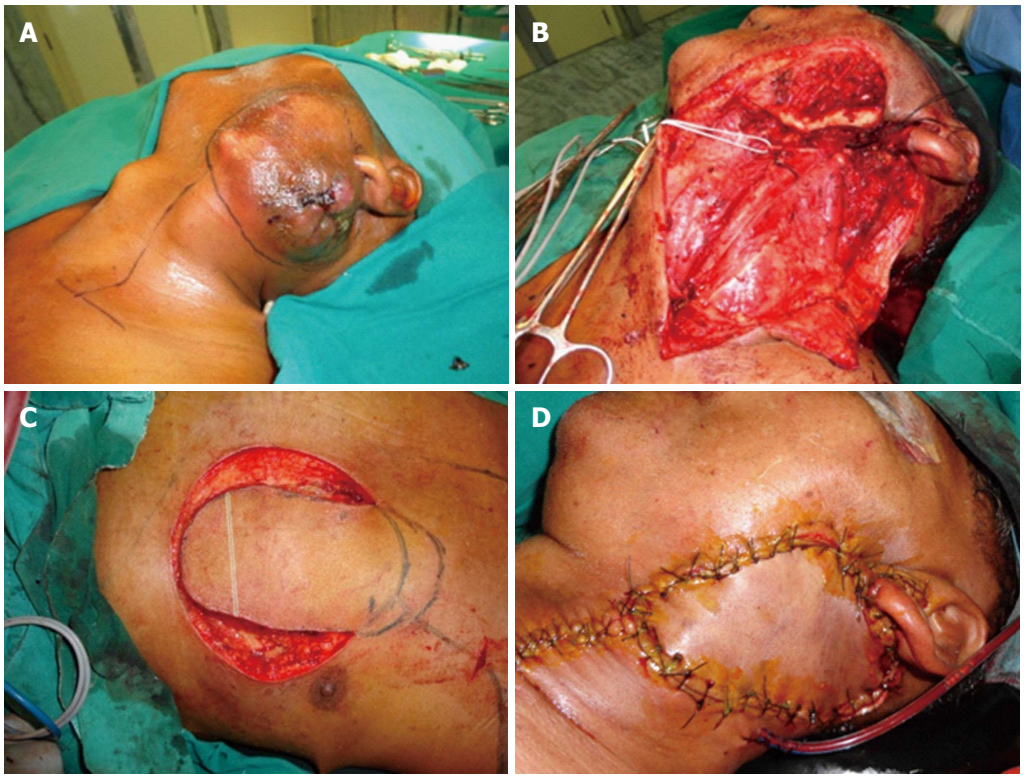

Figure 5 Reconstruction of large parotid skin defect (Patient 6). A: Tumor parotid with skin involvement; B: Post-ablative defect; C: Skin paddle with marked line representing approximate level of inferior border of pectoralis major muscle; D: Immediately following reconstruction.

of the skin paddle to prevent shearing and loss of blood supply. Further harvest must be done gently, avoiding tension or torsion of the pedicle. Similarly, when muscle around the pedicle needs to be thinned, care must be taken to avoid thermal or crush injury to the pedicle. Provided these principles are strictly adhered to, we believe an inferiorly extended skin paddle can be safely harvested, avoiding previously reported complications with the pectoralis major flap[8-10]. Further, the distance between the lower skin margin and the inferior border of the pectoralis major muscle has been limited to 5 cm or less in this report. Future studies may assess whether further extension of the skin paddle inferiorly is feasible.

The indications for the extended flap in this report were large defects of mucosal and/or skin surface, high defects involving soft palate or extending up to and beyond the temporal line, and complex, multi-dimensional intraoral defects requiring tension-free circumferential closure. In our assessment, the traditional pectoralis major flap would have been insufficient for tension-free resurfacing of these defects. Even though a few flaps in this series had a relatively smaller skin paddle size, the extended flap conferred the advantage of superior reach, rotation and contouring for high and complex defects. We believe the extended flap overcomes several of the limitations imposed by the traditional pectoralis major flap by conferring a larger skin paddle for big defects and superior reach for high defects, thereby minimizing tension and overall compromise of blood supply to the flap. It is plausible that some of the complications reported previously with the pectoralis major flap[8,9] might have been avoided by utilizing the extended flap. Done in an appropriate manner as outlined here, the extended flap increases the versatility of pectoralis major myocutaneous flap in head and neck reconstruction.

Post-ablative head and neck defects that involve large surface areas of the skin and/or mucosa, or extend high to involve the soft palate or up to and beyond the temporal line, are challenging to reconstruct. In this report, the extended pectoralis major myocutaneous flap has been shown to be ideal for repairing these large and complex defects. Further larger studies are required to confirm and expand our findings.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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15 Morse SS. Factors in the emergence of infectious diseases.

Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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