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HEALING OF TYMPANIC MEMBRANE PERFORATIONS: AN EXPERIMENTAL STUDY

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To my beloved parents
and
my cousin Akhtar Khan

ABSTRACT

Most acute tympanic membrane perforations heal spontaneously without any residual complication, whereas a fraction transforms into a chronic perforation. The current treatment of a chronic perforation is surgical repair, which is a costly, time consuming and uncomfortable procedure particularly for the pediatric patients. The objective of this thesis is to evaluate the underlying mechanisms of the reparative process in acute and chronic perforations. This is done by monitoring the structural and mechanical properties of the tympanic membrane in various situations during healing. Furthermore, an aim is to test the efficacy of stem cells as a healing adjuvant both in acute and chronic perforations.

On otomicroscopic examination the reduction of the laser perforation size was evident between day four and five after myringotomy. Histological analysis revealed thickening of the tympanic membrane at and around the myringotomy site at two and four weeks post-myringotomy in light microscopic sections. Transmission electron microscopic sections revealed a five-fold thickness increase in the lamina propria. Proliferation of fibroblasts and accumulation of large amounts of extra-cellular substances suggests an attempt of the body's reparative mechanism to revert the damage. The lamina propria is thickened still at six months after myringotomy. The fibers of the lamina propria are loosely packed with seemingly unorganized orientation. These findings suggest that the healing process is not yet finished after half a year. A striking histological feature was the larger thickening increase in the lamina propria of the tympanic membranes that were treated with stem cells, as compared to the untreated. Growth factors or other secreted substances from the stem cells might have influenced the proliferation of fibroblasts and the production of extra-cellular substances to contribute to this accentuated thickness.

An in vitro model in the rat was developed and adjusted for moiré interferometry measurements in order to assess the stiffness of normal and healed myringotomized tympanic membranes. Moiré interferometry is an optical, non-contacting technique by which the shape and pressure-produced displacement of an object can be measured. This method provides a full-field, 3-dimensional, overall stiffness change along with local stiffness variation of different portions of the tympanic membrane.

The stiffness of myringotomized and healed tympanic membranes is almost normal already at two weeks after myringotomy. A huge over-production of fibers during the proliferative phase of the healing seem to limit the damage caused by the myringotomy and thus provide extra strength to this area of reorganization. Long-term measurement of the myringotomized and healed tympanic membranes showed slightly reduced strength as compared to normal.

Acute perforations treated with mouse embryonic stem cells did not show any enhanced healing as compared to untreated. Teratoma, which is a known complication to embryonic stem cell treatment, was not detected in any of the ears after a follow-up time of six months. The use of human mesenchymal stem cell treatment on chronic perforations showed better healing as compared to the untreated. Thus, it appears to be of great importance to continue to study this type of stem cells in the treatment of chronic tympanic membrane perforations.

Key words: Embryonic stem cells, Healing, Immunosuppressive, Laser, Mesenchymal stem cells, Moiré interferometry, Myringotomy, Perforation, Tympanic membrane

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Rahman A, Hultcrantz M, Dirckx J, Margolin G, von Unge M. Fresh tympanic membrane perforations heal without significant loss of strength. *Otol Neurotol* 2005: Nov 26(6); 1100-6

II. Rahman A, Hultcrantz M, Dickx JJ, von Unge M. Structural and functional properties of the healed tympanic membrane : a long-term follow-up after laser myringotomy. *Otol Neurotol* 2007: April 11 (Epub ahead of print)

III. Rahman A, von Unge M, Olivius P, Dickx JJ, Hultcrantz M. Healing time, long-term result, and effects of stem cell treatment in acute tympanic membrane perforation. *Int J Ped Otolaryngol* 2007: (In press)

IV. Rahman A, Hultcrantz M, Olivius P, von Unge M. On the healing of chronic tympanic membrane perforations and an evaluation of a chronic perforation model. *Audiol Neurootol* 2007: (submitted)

CONTENTS

LIST OF ABBREVIATIONS

ES	Embryonic stem cell
GFP	Green fluorescent protein
KTP	Potassium titanyl phosphate
kPa	Kilo Pascal
LM	Light microscope
MI	Moiré interferometry
ME	Middle ear
MSC	Mesenchymal stem cells
OsO_4	Osmium tetroxide
PBS	Phosphate buffer saline
SD	Standard deviation
TEM	Transmission electron microscope
TM	Tympanic membrane

1 INTRODUCTION

The tympanic membrane (TM) plays an important role in the physiology of hearing as well as in the pathophysiology of inflammatory middle ear diseases. The TM perforation is a common sequel of long-standing and recurrent inflammatory middle ear diseases. The TM has a tremendous capability to heal after injury while most of the TM perforations heal spontaneously. Despite this high healing capability, the closing of chronic TM perforations is still a crucial issue for otolarygologists in terms of finding a simple method to heal it. The chronic TM perforations significantly impair the quality of life for millions of patients. The anatomy and the hearing physiology of the TMs have been well documented during the past several decades. Sufficient knowledge about the mechano-acoustic behavior of myringotomized and healed TM is still lacking. In the search for the simple treatment of chronic TM perforations it is important to evaluate the mechanical along with the structural outcome.

1.1 THE EAR

The mammalian ear is the organ of hearing and balance. Only vertebrates have ears and among them the mammals ear are the most complex and highly developed. Invertebrate animals lack ears but have other organs that serve similar functions. The ear consists of three parts: the outer, the middle and the inner ear (Fig.1). The outer ear is composed of an auricle (or pinna) and an external auditory canal. The auricle projects from the side of the head and helps to collect the sound waves in the air. The external auditory canal is a tubular passageway, which in man is approximately 2.5 cm long and runs from the auricle to the eardrum (or TM). It is lined with delicate hairs and small sebaceous glands which produce a wax-like secretion called cerumen. The middle ear is a narrow, air-filled chamber that consists of the tympanic cavity, the Eustachian tube and the mastoid air cell system. The tympanic cavity contains three auditory bones, the ossicles: malleus, incus and stapes. The malleus has a long process (handle or manubrium) that is attached to the TM. The incus acts as a bridge between the malleus and the stapes. The footplate of the stapes fits into a bony orifice called the oval window which fronts the inner ear. The inner ear (or labyrinth) lies deeply in the temporal bone and consists of the cochlea, the vestibule and the three semicircular canals. The cochlea is responsible for hearing and the vestibule for balance. The bony cochlea is a coiled tube and along its entire length it is divided into three fluid-filled compartments; the scala vestibule, the scala media and the scala tympani. The vestibular membrane (or Reissner's membrane) separates the scala media from the scala vestibule. The hearing organ (or organ of Corti) is located on the basilar membrane which separates the scala media from the scala tympani. The vestibular labyrinth consists of a complex series of interconnecting membranous ducts and sacs which contain the vestibular sensory epithelium.

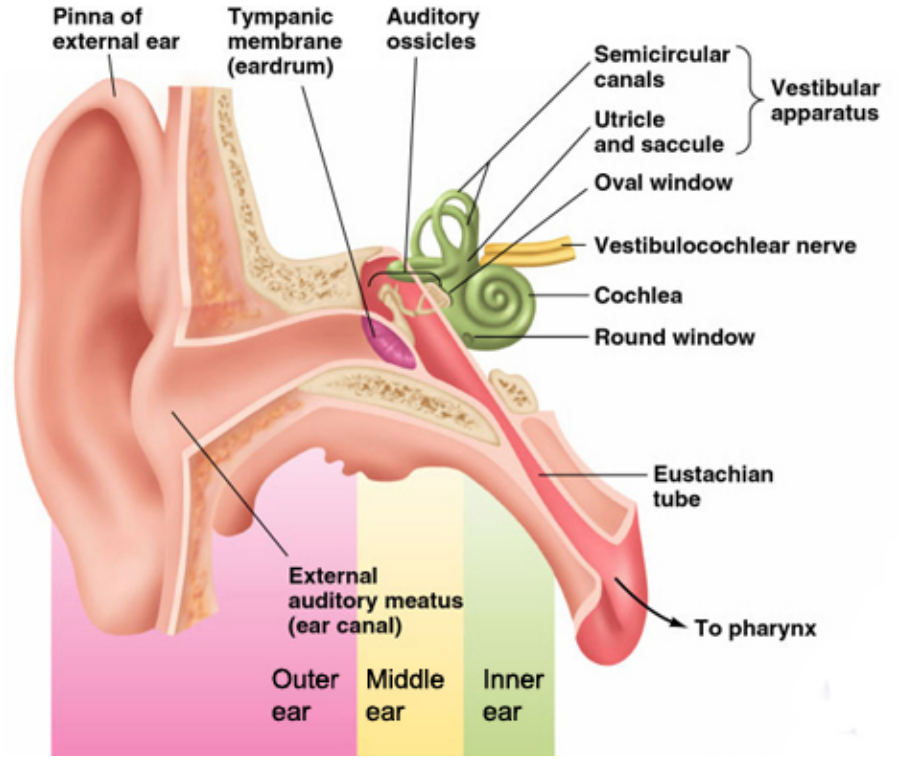

Figure 1: Anatomy of the human ear showing the outer, middle and inner ear (adapted from http://www.colorado.edu/kines/Class/IPHY3430-200/image/ear.jpg)

1.2 THE TYMPANIC MEMBRANE

The development of the human TM is a complicated procedure. It is formed at four to five weeks of gestation by the union of two layers: an outer one, that is derived from a primitive ectodermal meatal cord and an inner one that is derived from the endodermal tubotympanic recess (first pharyngeal pouch). At eight weeks of gestation, mesodermal tissue grows in between these two layers to form another fibrous layer called lamina propria. From the 9^{th} week onwards, the tympanic ring starts to form and is completed at sixteen weeks of gestation. At birth the TM is almost adult-sized and positioned in a nearly horizontal way (Anson BJ, 1980).

1.2.1 Anatomy

The human TM is a thin, translucent, cone shaped diaphragm, slightly broader superiorly than inferiorly, forming the central portion of the lateral wall of the tympanic cavity. It´s longest diameter is 9-10 mm from the posterosuperior to the anteroinferior quadrant and it´s shortest diameter is 8-9 mm perpendicular to this direction, forming an angle of approximately 55^0 to the floor of the meatus, with a total area of approximately 64 mm^2. The greater part of it's circumference is thickened to form a fibrocartilaginous ring called the annulus, which is attached to a groove of the tympanic bone called the tympanic sulcus. This sulcus is deficient superiorly, where instead the bone has the formation of a notch (notch of Rivinus). From the ends of this notch two fibrous bands called the anterior and the posterior malleolar folds run centrally to the

lateral process of the handle of malleus, which lies within the tympanic membrane. The TM consists of two parts, the pars tensa and the pars flaccida (Fig. 2). The pars flaccida is a small, triangular region above the malleolar fold and it does not have any tympanic annulus at its margins. The rest of the TM below the malleolar fold is called pars tensa. It is taut and concave when seen from the ear canal, with the maximum depression at the inferior tip of the malleus handle, the area known as umbo. When seen from the middle ear side it looks gently curved with a slight convexity. The thickness of the pars flaccida measures 0.03 to 0.23 mm and the pars tensa measures 0.03 to 0.09 mm.

The TM consists of three layers: an outer epithelial layer, (or epidermis), which is continuous with the skin of the external auditory canal, a middle, mainly fibrous layer (or lamina propria) and a mucosal layer which is continuous with the lining of the tympanic cavity. The lamina propria consists of connective tissue and is considered to constitute most of the mechanical properties of the TM. The predominant feature of the lamina propria, in both the pars tensa and the pars flaccida is the presence of collagen fibers. The fibers of the outer (or lateral) layer of lamina propria are radial in orientation. The fibers closest to the epithelial layer are usually in direct contact with the basement membrane of the epidermal layer, although in some places a thin layer of loose connective tissue intervenes. The inner (or medial) layer fibers are in a circular arrangement. There are also some parabolic, transverse and crescentic fibers present in the inner layer. A loose connective tissue layer, containing fibroblasts, macrophages, nerve fibers and capillaries lies between the inner layers of the lamina propria and the inner mucosal layer. Neither the capillaries nor the nerves appear to penetrate or enter the mucosal layer. The mucosal epithelium of the pars tensa varies in height from a low simple squamous or cuboidal to a pseudostratified columnar epithelium.

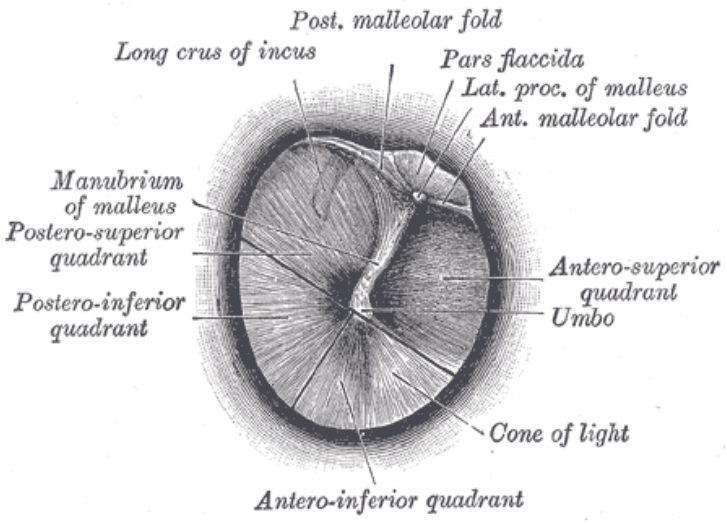

Figure 2: Different parts of the human tympanic membrane (adapted from www.bartleby.com)

1.2.2 Vascular Supply

The arterial supply of the TM is complex and arises from branches supplying both the external auditory meatus and the middle ear. The vessels are found only in the connective tissue layers of the lamina propria. Within this layer there appears to be a peripheral ring of arteries connected by radial anastomoses, with one or two arteries that run down each side and around the tip of the handle of malleus. The lateral side is supplied by the deep auricular branch of internal maxillary artery and the medial side by the stylomastoid branch of the posterior auricular artery and the anterior tympanic branch of internal maxillary artery. Several small twigs of the middle meningeal artery are also contributing to the arterial supply. The venous drainage returns to the external jugular vein, the transverse sinus, dural veins and the venous plexus around the Eustachian tube.

1.2.3 Nerve supply

Like the blood vessels, the nerve runs similarly in the lamina propria. The lateral surface is innervated by the auriculo-temporal branch of the trigeminal nerve and the auricular branch of the vagus nerve, whereas the medial surface is innervated by the tympanic branch of the glossopharyngeal nerve. The variations and overlaps are considerable, but both the vascular supply and the innervation are relatively sparse in the middle part of the posterior half of the TM.

1.3 THE RAT MIDDLE EAR

Guinea pigs, mice and rats are the most commonly used animals for experimental models in otolaryngology. Extensive knowledge about the middle ear anatomy of these animals is valuable. The Sprague Dawley rats have been used in all of our experiments because they are inexpensive and the middle ear structure is easily approachable. Almost all human genes known to be associated with disease, have orthologs in the rat genome, and recently the rat genome have been mapped successfully (Consortium, 2004), why this animal has now become even more valuable as an experimental tool in middle ear research. All the anatomical structures of the human middle ear are found in the middle ear of the rat. One published report even describe the middle ear morphology of the rat as exhibiting a micro-type organization (Fleischer, 1978). In rat, the area of the TM is approximately 11 mm^2 (Zimmer et al., 1994) whereas in humans it is approximately 64 mm^2. The relative sizes of the pars tensa and pars flaccida are different in humans and rats. The human pars flaccida is quite small compared to the total size of the TM, whereas in rats, the pars flaccida occupies between one quarter and one third of the TM area. The lamina propria of both human and rat TM showed the similar architectural feature and fiber arrangement (Schmidt and Hellstrom, 1991). The ciliated and secretory cells of the rat middle ear cavity are present in two different tracts (connecting epitympanum with the Eustachian tube), one anterior and one inferoposterior to the promontory (Albiin et al., 1986). The rat mucosa showed striking similarities to that of humans. The pars flaccida and the manubrial part of the pars tensa is supplied by the branches from the external carotid artery through the external auditory meatus. This vascular network lies beneath the squamous epithelium, close to mast cells and nerve bundles. Vessels originating from the tympanic cavity (probably a branch of the external carotid artery) supply the periphery of the pars tensa and are localized immediately beneath the tympanal epithelium (Albiin et al., 1985).
In terms of hearing, however, the frequency field is different in humans and rats.

1.4 BASIC HEARING MECHANISM

The hearing is a completely mechanical process and the system is solely based on physical movements. Sound is a series of vibrations moving as waves through air, gases, solids or liquids. The pinna serves to catch the sound waves. The pinna, along with the concha and the external auditory meatus has an influential effect on incoming sound, namely to increase the pressure at the TM in a frequency-sensitive way based on resonance. The sound waves passing through the external auditory meatus strike the eardrum causing it to vibrate. These vibrations are then transmitted through the chain of ossicles to the oval window. The middle ear couples sound energy to the cochlea and serves to match the impedance of the air to the much higher impedance of the cochlear fluids. As the vibrations pass from the relatively large area of ear drum to a smaller area of oval window, their force become concentrated and the sound becomes amplified. When the sound vibration reaches the stapes, it pushes the oval window forth and backwards, creating a motion inside the fluids of the vestibular and the tympanic canal. The middle ear also serves to apply sound preferentially to only one window of the cochlea, thus producing a differential pressure between the windows, required for the movement of the cochlear fluids. The altering changes of pressure in the fluid of the canals cause the basilar membrane to move. The organ of Corti contains the sensory cells with their hair like projections, which is part of the basilar membrane also moves, causing bending of the hairs. The movement of the hair cells and hairs send an electrical impulse through the cochlear nerve to the cerebral cortex where the auditory center interprets the impulses.

1.5 TYMPANIC MEMBRANE AND HEARING

In an open field, a fraction of the incoming sound is reflected off the head. A sound source coming from the opposite side of the head will result in shadowing around the head and the sound amplitude can be drastically reduced. The pinna is acting like an ear trumpet, collecting sound from a large area and concentrating it to a smaller area of the auditory meatus. In this way, the total energy reached in the TM is increased. The sound pressure at the TM changes in response to a resonance in the external auditory meatus in a frequency-selective way. If a tube is one-quarter of a wave length long, and one end is open and the other end is closed, the pressure will be low at the open end and high at the closed end when placing the tube in a sound field. This phenomenon is observed in the human external auditory meatus at a frequency of about 3 kHz. Here, the resonance adds 10-12 dB at the TM (Shaw, 1974). Different resonances increase the sound pressure in different frequencies. The broad resonance arising in the pinna is adding about 10 dB at 5 kHz. The total effect of reflections from the head and the pinna and the various external ear resonances is adding 15 -20 dB to the sound pressure over the frequency rate from 2 to 7 kHz (Stinson and Khanna, 1989).

The sound waves that strike the TM are partially reflected back into the ear canal and partially transmitted across the TM. Sound waves can be transmitted to the inner ear through two pathways. One is vibrating waves of the TM passing through the ossicles to the oval window and in the other, sound waves cross the TM directly and enters the round window. The middle ear serves as a transformer to match the impedance of the air to the much higher impedance of the cochlear fluids. The optimal power transfer is possible only when the impedances of the media involved are matched. The most obvious transformation is taking place due to the larger areal ratio of the TM and the stapes footplate. Like the diaphragm of a loud speaker, the entire surface of the TM is

not free to vibrate, but even considering this factor the ratio is approximately 13:1. Thus the force of the sound wave acting over the TM piston is funnelled to the much smaller footplate area, gaining the sound pressure amplification. A hologram of the TM in motion implies that the curvature of the TM is the principal component of the transformer, rather than the TM and foot plate ratio.

1.6 MOIRE INTERFEROMETRY

Moiré interferometry (MI) is an optical, non-contacting technique for measuring the shape and deformation of delicate biological structures. By using interferometric techniques it is possible to record the fringes of equal height, equal displacement amplitude or equal vibration amplitude on a diffusely reflecting object from a distance without contacting the recorded object. Several optical measurement techniques are available to study the vibration, shape and deformation of delicate structures. Daily-life events like sneezing, coughing, using elevator or diving in a pool causes short-standing elevation of middle ear (ME) pressures up to several kilo pascles (kPa) (Sakikawa et al., 1995). Long-standing variations of ME pressures upto several kPa is also observed under normal situations (Tideholm et al., 1996). The vibration pattern of the TM *in vitro,* both human (Tonndorf and Khanna, 1972) and cat (Khanna and Tonndorf, 1972) has been investigated by using holographic interferometry. *In vivo* measurement of human TM vibration was performed by electronic speckle pattern interferometry (Lokberg et al., 1980). MI is a suitable technique for measuring the TM shapes (Fig. 3) and deformation (Fig. 4) caused by static ME pressures. During measurements, a set of evenly spaced grating lines are projected onto the object surface from a distance. The shape of it's surface modulate the grating. The resulting deformed line pattern is then recorded and stored in a computer. By optical interference between the deformed and non-deformed line pattern, moiré fringes are created. These form a set of contours of equal height and thus provide a topographical map of the object. The displacement is produced by applying pressure gradients across the TM. The first image is recoded at an original state of the object whereas the second image is recorded at deformed state of the object. Deformation is obtained by reconstruction of the object shape from these topograms and finally calculating the difference between these two shape measurements. To obtain the best results from MI, it is essential that the recorded surface reflects light diffusely. The reflectiveness of an object can be improved by coating the surface with a physiologically harmless paint such as white china ink.

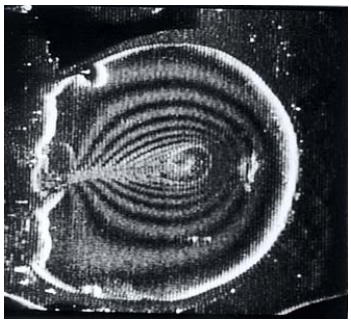

Figure 3: Shape recording of a normal TM at rest by moiré interferometry

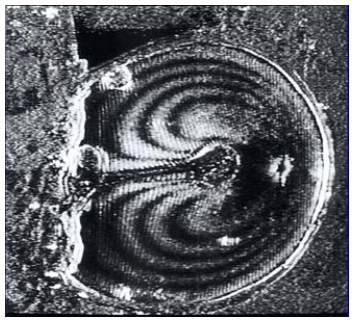

Figure 3: Displacement recording of a normal TM against a pressure load by moiré interferometry

1.7 TYMPANIC MEMBRANE PERFORATION

1.7.1 Causes

The acute TM perforation may arise from trauma, infection, pressure and medical care. Trauma associated injuries may arise from instruments such as cotton swabs, bobby pins, and sticks, skull fractures, acid burns and welding or metalworking slag burns. Infection is the principal cause of TM perforations and the most common infectious cause is acute otitis media. Rarely, acute necrotic myringitis with fulminant necrosis (due to beta-hemolytic streptococcus) leads to a perforation. Pressure-induced perforations can occur after loud noises or explosions, open palm trauma (slapping), or changes in ambient pressure that occur during flying or underwater diving. Increased pressure (from descent in an airplane or diving under water) causes the ambient pressure to become greater than that of the middle ear. If the Eustachian tube does not equalize the pressure, the TM may bulge inward and rupture. Iatrogenic causes during medical care such as inexpertly performed irrigation of the ear canal for wax removal, ventilating tube insertion and emergency myringotomy (for hyperbaric therapy).Other TM perforations are classified as chronic, and most are the result of recurrent ear infection.

1.7.2 Clinical impact

Patients present with symptoms such as pain, bleeding or discharge, tinnitus and hearing loss. Ear canal infections can also cause purulent discharge, but usually in lesser amount. Perforations uncomplicated by infection or cholesteatoma are not usually painful. When there is hearing loss, the size of the perforation generally correlates with the degree of the hearing loss, but the location is also a factor. Severe hearing loss may result if significant trauma (as in skull fractures) disrupts the ossicles or involves the inner ear. Acute TM perforations usually heal spontaneously. Chronic perforations require medical assistance because the patients suffer from impaired hearing, draining ear, sometimes tinnitus, and vulnerability of entering water into the ear during swimming or bathing, which might lead to infection.

In case of an acute perforation some observation time is recommended before surgical intervention. Surgery may consist of the simple procedure of fat-plugging, which has a limited success rate, or regular middle ear surgery, which still has some percentage of failure and has some risk of complications.

1.8 WOUND HEALING

Healing of wound is the body's natural process of regeneration which leads to restoration of tissue integrity, architecture and function. A series of events takes place during the process in an orderly fashion and some events overlap in time. It is roughly divided into three phases, *the inflammatory*, *the proliferative* and *the wound contraction* (*or remodeling*) phase.

The inflammatory phase starts immediately following tissue injury. The injury initiates a vascular and cellular response that clears the wound of devitalized tissue and foreign materials and creates an environment for tissue healing and regeneration.

Vascular injury due to trauma initiates an intense vasoconstriction of 5 to 10 minutes which help in hemostasis. This is followed by vasodilation which becomes pronounced approximately 20 minutes after the injury and is accompanied by an increase in capillary permeability. When the blood components spill-out in to the site of the injury, the platelets come into the contact with exposed collagen and extracellular matrix. This contact triggers the platelets to release clotting factors, essential growth factors and cytokines such as platelet derived growth factors (PDGF), transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF), which helps to achieve hemostasis. Following hemostasis neutrophils come to the scene to begin the critical task of phagocytosis to remove bacteria, foreign particles and devitalized tissue. At the end of this phase macrophage enter into the wound site and continue the process of phagocytosis.

The proliferative phase begins once the wound site is cleaned out, fibroblasts migrates to the site within two to three days and deposit new extracellular matrix. The early extracellular matrix includes fibronectin and hyaluronate, which serves as a scaffold upon which fibroblast can migrate and adhere. Fibroblast produces a variety of substances that are essential for wound healing, including glycosaminoglycans and collagen. The four important glycosaminoglycans include hyaluronic acid, chondroitin-4-sulfate, heparin sulfate and dermatan sulfate. These form together an amorphous material called "ground substance" that plays an important role in aggregation and deposition of collagen fibers. During this phase, the proliferation of collagen continues rapidly approximately three weeks until a hemostasis is achieved. After this time period the rate of collagen degradation equals that of collagen synthesis. The amount of collagen deposition during this proliferative phase determines the tensile strength of a healed wound. Angiogenesis occurs concurrently with this fibroblastic phase to provide the metabolic needs and if this process of new capillary formation fails, fibroblast migration is halted and the healing fails to proceed. In the first two to three days after injury fibroblast activity is mainly confined to the migration and proliferation rather than collagen synthesis. After this period, fibroblast starts to synthesize and secrete measurable amount of collagen. Fibroblasts are the main source of collagen and extracellular connective tissue. The new collagen matrix are then covalently cross-linked and organized to enhance the tensile strength of the wound. The epithelial cells immediately adjacent to the wound are stimulated to begin migration when they lose the contact inhibition. These migrating and proliferating epithelial cells progress towards the wound to cover it until the edges meet. At this stage, further proliferation and migration ceases, due to the phenomenon of contact inhibition. At one week following the injury collagen synthetic activity reaches its maximum rate, and immature collagen fibers become evident in histological preparation.

The contraction or remodeling phase starts when the level of collagen production and degradation reached a plateau. Remodeling of collagen fibers into a more organized way occurs during this maturation phase. This maturation process can continue for a year or more depending on the condition of the wound. During this process the wound progressively continues to increase in tensile strength. There is a lag phase of 10 to 14 days, when the tensile strength of the fresh wound remained weak. Following this period, there is a rapid increase in tensile strength over the next four weeks, when the wound gain 70% of the strength of undamaged tissue. A slow steady phase is then approached and approximately two years post-injury, the wound reaches about 80% of the normal strength and the healed wound never exceeds this value (Stadelmann et al., 1998). During this whole process of repair, there are numerous cell-signaling events that takes place to control and maintain the process efficiently.

The healing of the TM is not similar to that of other cutaneous structures. Immediately after trauma, the squamous epithelium at the edge of the perforation becomes hyperplastic and produce excessive keratin (Stenfors et al., 1980). These proliferative epithelial cells along with keratin first try to cover the wound. The epidermis close the perforation in the direction of surface migration. Secondarily the fibrous reaction takes place and in-growing connective tissue laid down beneath the epithelial covering (Johnson et al., 1990). The closure of the connective tissue defect is always preceded by the healing of the epithelial layer. This mechanism differs from the normal healing process of other cutaneous wounds where the squamous epithelium migrates over a newly formed granulation tissue (Boedts and Ars, 1977).

1.9 TREATING TM PERFORATIONS

The first attempt to close a TM perforation was reported by Dr. Banzer (Banzer, 1640). He inserted a small tube of elkhorn covered with a pigs bladder into the ear to replace the ruptured area. Later, Dr. Leschevin (Leschevin, 1763) and Dr. Autenrieth (Autenrieth, 1815) proposed the use of an "artificial drum" regarding the sound conduction in the normal middle ear. Berthold performed the first surgical closure of a TM perforation with a full thickness free skin graft (Berthold, 1878). Berthold and Ely (Ely, 1881) argued about who did the first closure of a perforation by a skin graft. Berthold used his technique for at least ten years but was unable to persuade his colleagues to practice the treatment. After this, there was a long period of time until the middle of the twentieth century where no attempts to close TM perforations was reported. The TM and middle ear surgery entered into a new era when Zöllner (Zöllner, 1951) presented his myringoplasty technique after 1950. Full-thickness or split-thickness skin grafts were used as grafting materials at that time. However, recurrent perforation, cholesteatoma and excessive desquamation prompted the search for other grafting materials. A significant number of reports concerning the use of different tissues and materials for grafting purposes, ranging from autografts to homografts, heterografts and artificial materials have been performed with variable success rate. Temporalis muscle fascia was first used in myringoplasty by Ortegen in 1959, and one year later L. Storrs introduced the same grafting material in the United States, after which it has been used widely and has become one of the most popular grafting materials till to date. Closing TM perforations by using fat (or fat plugging) was first attempted by Ringenberg (Ringenberg, 1962). In 1964, this method of closure of small TM perforations was confirmed by Sterkers (Sterkers, 1964) with the use of compressed abdominal fat and was later reconfirmed by Terry (Terry et al., 1988) and Gross et al.(Gross et al., 1989). The first attempt to develop a simpler, cost-effective and easily applicable technique which could be used in an office setting, Blake introduced paper-patch graft in 1887. In this method the paper-patch guides the migrating epithelium as a scaffold from the perforation edges. In recent years, various substances like hyaluronic acid (Chauvin et al., 1999), growth factors (Ma et al., 2002), pentoxyfylline (Lim AA, 2000), heparin (Hellstrom and Spandow, 1994) were used for healing tympanic membrane perforation.

1.10 STEM CELLS

Stem cells are unspecialized precursor cells that can renew themselves for longer periods of time through cell division. They can differentiate into specialized cells in response to appropriate signals. Generally, stem cells are classified as either embryonic stem (ES) cells or tissue-specific (adult) stem cells. ES cells are generally regarded as pluripotent as shown by Mintz (Dewey et al., 1977). Here, teratocarcinoma cells were isolated, genitically marked and implanted into the blastocyst of a foster mother. The resulting mice were normal, yet had chimeric mixtures of teratocarcinoma and wild-type cells in virtually every tissue of their bodies. ES cells are derived from the inner cell mass of blastocysts, and possess an unlimited potential for self renewal and the capacity to differentiate into all kinds of somatic cell types *in vitro* (Evans and Kaufman, 1981) and *in vivo* (Bradley et al., 1984) as well as germ cells (Hubner et al., 2003). ES cells can be maintained in an undifferentiated state indefinitely without losing their differential potential. Culture over long periods of time may give rise to karyotypic abnormalities (Draper et al., 2004). Adult stem cells are unspecialized cells found in differentiated tissues that can self renew for a longer period of time and differentiate into specialized cell types of tissue in which they reside.

A recent study (Fraidenraich et al., 2004) gave an important breakthrough by transplanting human ES cells into mutant knock-out mouse embryos with lethal cardiac defects. The study demonstrated that the rescue of lethal defects can be achieved either through injection of wild-type ES cells directly into the mutant embryos or by injection into the maternal circulatory system. Histological analysis revealed that the transplanted ES cells do not give rise to functional cardiac lineages within the defective embryonic heart. However, they provided indirect evidence that the therapeutic effect might be functioning due to the secretion of various factors from the transplanted cells which could act either locally within the defective heart, or at a distance via the maternal circulation.

The immunological rejection is the most formidable challenge in using ES cells in transplantation therapy. Formation of teratoma is another common complication while using such cells. The potential utility of ES cells as cellular catalysts to promote biological repair and regeneration in transplantation therapy still embedded with as much technical challenges, as the promise it holds.

Multipotent mesenchymal stem cells (MSC) are capable of differentiating into numerous cell types, including fibroblasts, bone, cartilage, muscles (skeletal and smooth) and brain cells. Studies have been reported that adult MSCs, when injected into animals, are capable of homing to a site of injury and restoring tissue function (Dennis JE, 2004). MSC are relatively easy to isolate from small aspirates of bone marrow such as the iliac crest (Haynesworth et al., 1992), vertebra (D'Ippolito et al., 1999), tibial and femoral shafts (Oreffo et al., 1998; Murphy et al., 2002) and are easily expanded in culture while maintaining phenotypic stability. Bone marrow derived MSC are capable of trans-differentiating into multiple cell types and to produce tissue repair factors. Therefore these cells provide an alternative to the ES cells for the treatment of wounds. Several reports about the successful use of these cells as a restorative therapy of stroke (Chopp and Li, 2002), myocardial infarction (Orlic et al., 2001), and osteogenesis imperfecta (Horwitz et al., 1999) encouraged scientists to use MSC in complicated wounds to promote healing.

2 AIMS OF THE STUDY

The overall purpose of the study was to find a simple, easily executed, office technique that could replace the conventional surgical treatment for repairing chronic tympanic membrane perforations. Based upon the above goal, the present study included the following aims:

- To adjust a moiré interferometry set-up for stiffness and displacement measurements on the Sprague-Dawley rat and CBA mice temporal bone, and to ascertain the degree of functional and structural restitution of the healed TM, following a traumatic fresh perforation.

- To determine the stiffness and strength of a myringotomized and healed TM, at a long-time follow-up after the perforation.

- To determine the healing enhancing capability and long-term complication of ES cells applied in acute TM perforation models.

- To evaluate a chronic perforation model, the healing of the perforation and the use of MSC to heal chronic perforations.

3 MATERIALS AND METHODS

(For detailed description, please see the individual paper)

3.1 ANIMALS

All animals used in this thesis were female Sprague-Dawley rats, aged between 8 and 12 weeks, weighing between 250 and 300 grams. The animals were purchased from the breeding facility of Biomedicinsk Centrum, Uppsala. They were free from middle ear infections as judged by otomicroscopic inspections and did not show signs of any other health problems during the experiments. The animals were housed in an animal facility, in groups of five animals per cage with free access to food and water on an artificial light and dark cycle (12/12 h). All animal experiments were approved by the ethical committee of Karolinska Institute (approval number N 298/03, N 67/02 and N 344/02).

3.2 ACUTE PERFORATION MODEL – LASER MYRINGOTOMY

Perforations of different diameters (0.2, 0.4 and 0.6 mm) were made in the TM by KTP laser beams directed through laser fibers. Single pulses of 1 watt with durations of 0.5 seconds were used in each application. The perforations were made at the postero-superior quadrant behind the handle of malleus with a precise accuracy. Thus the perforations were standardized with the chance of highest reproducibility.

3.3 CHRONIC PERFORATION MODEL

After laser myringotomy a solution of 1% hydrocortisone was instilled around the perforation for 10 consecutive days. Application of hydrocortisone for 1, 3 and 7 days was also tried.

3.4 STEM CELLS AND IMMUNOSUPPRESSIVE DRUG

Tau-tagged GFP labeled mouse ES were collected from the laboratory of Professor John O Masson, department of Biomedical Sciences and Center for Developmental Biology, Edinburgh, United Kingdom. The cells were dispersed in normal saline giving a solution of 1×10^4 cells per ml in each application.
Human MSC were collected from the laboratory of Katarina Leblanc, Karolinska Institute. A droplet of MSC dispersed in normal saline at the concentration of 1.2×10^6 cells per ml was instilled over the perforation in each application.
Injection cyclosporine and injection takrolimus (prograf) were used as an immunosuppressive agent in some stem cell treated animal groups.

3.5 GELATIN

Gelatin is a heterogeneous mixture of water-soluble proteins of high average molecular weights, present in collagen. The proteins are extracted by boiling skin, ligaments, tendons, bones etc. in water. The natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. Gelatin melts when heated and solidifies when cooled again. It forms a semi-solid colloidal gel when mixed with water. It is used in a wide range of food and non-food products. In this study it has been used as a suspending sheet for providing the stem cells to proliferate over the TM.

3.6 MOIRE INTERFEROMETRY MEASUREMENT

The MI measurements were performed on temporal bone preparations with normal TMs and myringotomized and closed TMs. After sacrificing the animals under anesthesia, the TMs were isolated along with the temporal bones, and soft tissues were removed. The tympanic bulla was opened widely and necessary dissection was performed to suit the specimen for moiré set-up. All the surgical procedures were performed under a constant steam from a water evaporator to prevent dehydration. A small cylindrical plastic pipe resembling the external auditory canal was attached by glue to the bony meatus of the external canal. The pipe and the ear canal were filled with white ink to improve the moiré fringe visibility when measured from the middle ear side. During shape measurements moiré fringes reflected the height of different portion of the TM based upon the annulus baseline. A series of static pressures was applied to the pipe-ear canal system, producing a pressure gradient across the TM. The pressure started from 0 daPa with steps of 50 daPa in 10 seconds intervals, increased up to 350 daPa and then decreasing back to 0 daPa. A series of negative pressures similarly with the positive one was also recorded. The peak displacement values were measured for each individual ear at each pressure level.

The whole TM surface was observed with a CCD camera (Sony AVE-D7CE). The observation direction of the camera had a fixed angle to the projection direction. The image of the TM overlaid with the projected grating lines was recorded. The video signal was digitized and stored in an image processor equipped with an image subtractor. When a sequence of pressure gradients was applied, the TM was displaced and the projected grating lines were thereby modulated. These newly recorded images were also digitized and were continuously subtracted in real-time from the first image of the TM without pressurization. Interference between these two images generates moiré fringes superimposed on the image of the TM. These fringes represent equal displacement and thus provide a 3-dimensional map of the TM displacement. The digital image was re-converted to analogue and displayed on a monitor and recorded on videotape. To compare results from different measurements each TM was placed in a similar position, i.e. with its annulus plane perpendicular to the observation direction. Thus the displacement component measured is the one perpendicular to the annulus. A TM shape image is produced in a similar way as the displacement images, with the difference that the two images used for interferometry are one recording of a flat glass plane and one recording of the TM in a non-pressurized state. The interference reflects the TM shape as compared to a flat surface.

3.7 MORPHOLOGICAL PROCESSING

3.7.1 Light microscopy

The TMs were collected with temporal bones immediately after sacrificing the animals in phosphate buffer saline (PBS). The specimens were then preserved in 2.5% glutaraldehyde for 24 hours for proper fixation. For decalcification, 0.1 M EDTA solutions were used and changed everyday until the bones were decalcified. The TMs along with temporal bones were dissected properly. Post fixation was performed with 1% osmium tetroxide (OsO_4). Gluteraldehyde stabilizes tissues by cross-linking proteins. OsO_4 reacts with lipids and certain proteins but also provide electron density to the tissue. The specimens were then dehydrated with graded ethanol solution (70%, 90%, 95% and 100%) and embedded in agar resin 100 and incubated at 40^0C for 24 hours and 60^0C for 48 hours for polymerization. The TMs were sectioned at 1 μm thickness with an ultrotome (LKB 2128). The sections were mounted on glass slides, stained with 0.1% toludine blue and examined under a Zeiss Axiolab Light microscope.

3.7.2 Transmission electron microscopy

Electron microscopy was used to analyze the ultra-structure of the TMs with respect to cellular composition and morphology. Tissue preparation for electron microscopy involves fixation, dehydration and embedding in low viscosity epoxy resins that polymerize to give a material suitable for the preparation of ultrathin sections. The main objective of this process was to stabilize and preserve the fine structural details of the fixed tissue as close to that of normal TMs in living animal. Glutaraldehyde and OsO_4 were used as the most effective fixatives for electron microscopy. Ultra-thin sections were cut on an ultratome (LKB Cryo Nova), collected and mounted on formvar-covered copper grids, and stained with uranyl acetate and lead citrate. The sections were examined and photographed in a transmission electron microscope (JEOL 1230).

3.8 STATISTICAL METHODS

Statistical analysis used in this thesis includes two-way ANOVA (SigmaStat, Sysstat software Inc., San Jose, California, USA). Difference was considered statistically significant when the p value was <0.05. Data on peak displacement of myringotomized control groups and treated groups are presented as mean values ± SD.

3.9 EXPERIMENTAL PARADIGM

In paper I, laser myringotomy was performed in the right TM leaving the left TM as a control. The mechanical stiffness and displacement measurement was performed at two and four weeks on both normal and myringotomized healed TMs. The mechanical stiffness was measured by MI at the University of Antwerp. A histological study was performed by LM and TEM.

In paper II, laser myringotomy was performed in the right TM leaving the left TM as a control. Otomicroscopic examination was performed twice a week for one month and at the end of the study at six month. Mechanical stiffness was measured by MI at the University of Antwerp for both normal and myringotomized and healed TMs. LM and TEM studies were performed for histological analysis.

In paper III, bilateral laser myringotomy was performed in animals divided into two groups, group A and B. In group A, the perforation size was 0.2 mm and a droplet of gelatin was applied around the perforation in both the left and the right TM. Mouse ES cells were applied only in the right TMs and left TMs served as controls. In group B, the perforation size was 0.4 mm, an immunosuppressive drug was injected for two weeks but gelatin was excluded from this group. Otomicroscopic examination was performed for both groups until the perforations were closed. LM and TEM studies were performed for both groups but mechanical stiffness measurements were done only for group B.

In paper IV, four studies have been described. A morphological study of acute TM perforations was performed in part 1. In part 2, the impact of the perforation size on the closing time was assessed by otomicroscopy. In part 3, a simplified method for producing a chronic perforation model was evaluated. In part 4, the effect of MSC treatment on the chronic perforation was investigated.

4 RESULTS

4.1 REGAINED STRENGTH TWO WEEKS AFTER PERFORATION (PAPER I)

In paper I, we have shown the comparative strength of a normal intact tympanic membrane with a closed myringotomized TM at two and four weeks after perforation. All the perforations were closed between 9 and 14 days after myringotomy according to the otomicroscopic examinations. The peak height was observed at the center of the anterior and the posterior half of the pars tensa in case of both the normal and the closed myringotomized TMs. In the normal control group the mean peak height was 6.8×10^{-4} m whereas in studied group it was 7.3×10^{-4} m. So the shape measurements did not show any significant difference between the two groups. The mean peak displacement for the normal control group at 350 daPa was 2.4×10^{-4} m with a standard deviation (SD) of 0.39×10^{-4} m and for the closed perforated group was 2.37×10^{-4} m with a SD of 0.79×10^{-4} m. The displacements difference between the groups was insignificant at the highest positive pressure levels, which means that both groups are equally resistant to such relatively large pressures. The mean peak displacement for the control group at – 350 daPa was 3.2×10^{-4} m (SD; 0.79×10^{-4} m) and for the studied group was 3.14×10^{-4} m (SD; 0.94×10^{-4} m). The displacement difference between the groups was minor and insignificant. The displacement was larger in the negative pressure sequence than in the positive one. Larger displacement was found in lower pressure zones near 0 daPa as compared with the higher pressure zones for both groups.

A hysteresis effect was observed in both groups and there was no significant difference in the hysteresis magnitude between the two groups. In the LM study, the layers of the TM were not clearly identified. In normal control ears the TM shows the same thickness and appears homogenous through its entire length. A slight thickness increase was found at both ends in the sections close to the annulus which is a normal histological finding in TM. Ears with closed perforations showed a local thickening at and around the site of the perforation but other regions of the TMs showed normal thickness. The TEM pictures revealed the detailed anatomical structure of the TM. A uniform lamina propria of about 2.4 μm thick with well oriented densely packed fiber bundles was found in normal TM. The outer and inner epithelial layers were lined by single stranded flat epithelium, with some keratin production found only in the outer epithelial layers. The total thickness of the pars tensa was approximately 5 μm. A significant thickness difference of lamina propria was observed in closed perforated TM compared with the control TM. A total thickness of 25 μm was noted which was five times higher than that of normal TM. The site of the perforation was identified by tracing the fiber bundles of lamina propria. This thickness was mainly contributed by the proliferating fibroblasts and large amounts of extracellular substances. This recruitment was mostly observed at the medial side of the lamina propria at or around the site of the perforation.

4.2 STRUCTURAL CHANGES OF HEALED TM AT 6 MONTHS (PAPER II)

The long term stiffness and strength of myringotomized and healed TMs has been measured and compared the results with normal controls. Otomicroscopic examination revealed that all the perforations were closed between 7 and 11 days after myringotomy. At six months, all the TMs appeared normal without any visible abnormalities. The displacement of the normal and the studied ears at + 350 daPa pressures showed similar displacement fringe patterns for both groups. The isolated control ear was investigated and plotted at each pressure point throughout the entire positive and negative pressure sequences. The studied ear was recorded similarly as the control one. When these two curves are incorporated, they exhibited an "S" shape pattern which is characteristic of biological soft tissues. The displacements were larger in the low-pressure zone, i.e. around zero pressure than in the positive and negative high-pressure zones. This is also a common finding in measurement of displacement versus pressure curves in soft tissues. The mean peak displacement for the control group at +350 daPa pressure was 2.6×10^{-4} m (SD; 0.99×10^{-4} m) and for the studied group was 2.75×10^{-4} m (SD, 0.50). At –350 daPa pressure the peak displacement for the control group was 2.45×10^{-4} m (SD; 0.41×10^{-4} m) and for the studied group was 3.35×10^{-4} m (SD; 0.25×10^{-4} m). Thus, the mean curve plots showed slightly higher displacements in myringotomized and healed TM as compared with their control counter part, especially in the positive pressure sequences. In the negative pressure sequences, however, the mean displacement curves for both groups were well within one standard deviation from 0 to – 150 daPa pressure. After this point the difference between these two groups was significantly increased according to the two way ANOVA analysis. The mean curve plot appeared as a smoothly rounded "S" shape.

A hysteresis effect was observed in all TMs of both the control and the studied group. There was no significant difference in the hysteresis magnitude between the two groups. In LM sections the pars tensa of the control TMs showed a uniform thickness of about 5 μm along its entire length with fairly homogenous appearance. In case of a myringotomized and healed TM, LM preparations displayed a persistent thickening at the site of the perforation, which in fact involved the entire half of the pars tensa. The other half of the pars tensa, posterior to the handle of malleus appeared normal. The TEM sections revealed a mean thickness of 4.3 μm, out of which the lamina propria contributed to the major part, around 3 μm. Densely packed fiber bundles in uniform orientation with some extra cellular substances were evident in the lamina propria. A mean fiber count of 75 per square micro meter was noted based upon on fiber counts at 10 different places of the TM. The lamina propria was lined by single stranded flat epithelia on both sides, the outer one producing keratin. The TEM sections of a myringotomized and healed TM displayed a significant thickening of the lamina propria, being around 15μm (total; 16.5μm) which is 5 times thicker than that of the control lamina propria. The thickness was observed widely around the myringotomy site. A mean count of traced fibers was 57 per square micro meter. Loosely organized fiber bundles were found in the lamina propria. Normally appearing outer and inner epithelia with intact basal lamina was detected.

4.3 STEM CELL TREATED AND HEALED TM STRUCTURE (PAPER III)

In both groups (A and B) the control side closed earlier than the ES cell treated side according to the otomicroscopic examination. Administration of immunosuppressive agent (group B) or gelatin (group A) did not show any impact on the healing. The TMs of group B closed earlier despite of larger perforations (0.4 mm) than those of group A (0.2 mm). The TMs of both groups were found free from infection or blood clot during the regular otomicroscopic inspection. At one month after myringotomy a ring shaped scar tissue was found at the myringotomy site in most of the TMs of both groups. These scars were no longer present at the end of the study at six months, and all the TMs appeared normal. Two treated TMs of group A were investigated for fluorescent labeled cells under fluorescence microscope and a faint staining was detected which could not be correlated to any specific cell or structure of the TM. In the LM sections the control TMs were thickened at the site of myringotomy at postero-superior quadrant whereas the anterior quadrant appeared completely unaffected. The treated TMs showed more thickening as compared with the controls and the thickening virtually covered the whole postero-superior quadrant. In this group, the anterior quadrant was unaffected too. In TEM sections the mean TM thickness of the control group was 28 μm whereas in the treated group it was 36 μm. The fiber arrangement of the lamina propria in both groups was disorganized with diverse direction. A characteristic finding of the lamina propria in both groups was a presence of abundant cells, especially fibroblasts. Edema was more pronounced in the treated group than in the control group.

Deformation measurement by moiré interferometry showed similar moiré fringe patterns for both control and treated groups. The mean peak displacement for the control group at +350 daPa pressure was 2.65×10^{-4} m (SD; 0.68×10^{-4} m) and for the studied group was 2.35×10^{-4} m (SD; 0.41×10^{-4} m). At -350 daPa pressure the peak displacement for the control group was 3.45×10^{-4} m (SD; 0.10×10^{-4} m) and for the studied group was 3.6×10^{-4} m (SD; 0.48×10^{-4} m). The largest hyeteresis effect for both the groups was found around 50 to 100 daPa in the positive pressure cycle. The mean curve plot of the control and the treated groups almost overlapped each other; imply that there was no difference in the stiffness of the TMs between the treated and untreated controls.

4.4 CHRONIC TM PERFORATIONS AND STEM CELL TREATMENT (PAPER IV)

4.4.1 Laser perforation start to close at around day five

Laser myringotomy of 0.4 mm diameter was monitored by otomicroscopy daily for 9 days in 9 animals. Otomicroscopic measurements after an hour and on day one, two, three and four did not show any obvious changes in perforation size as compared with the initial perforation diameter. On day five, however, the perforation of one TM was reduced to half of its original diameter. A reduced diameter of the perforation was observed consistently from day five onwards. The TM collected after one hour post myringotomy showed normal morphology in LM. At day one after myringotomy a slight thickening was observed at the central portion of the TM in the perforated quadrant. At day four the thicknesses was further increased towards the central portion of the perforated TM and to a lesser extent also on the peripheral part. At day eight, both edges of the perforation were significantly thickened. The non-perforated antero-inferior quadrant appeared normal throughout the healing process. TEM sections of TM isolated an hour after myringotomy displayed normal morphological architecture. At

one day after myringotomy, a large amount of epithelial proliferation was evident at the central part of TM close to the handle of malleus. A massive tissue wave of proliferating epithelia was detected on the lateral face of the TM towards the site of the perforation. At four days post-operatively, the peripheral edges of the lamina propria became thickened due to edema with infiltration of keratin and round cells in the proliferating epithelium. Some fibroblasts were also detected in the lamina propria. At eight days after the myringotomy thickened epithelial linings surrounded the edge of the lamina propria on peripheral side.

4.4.2 Perforation size and the healing time

Laser myringotomy of 0.2, 0.4 and 0.6 mm was tested in 3 different groups of rats. Some complete closure was first observed in two groups (0.4, 0.6) at day six. Closure of fifty percent or more was observed in two groups (0.2, 0.6) on day 10 whereas more than eighty percent was recoded in 0.4 mm group on the same day. Almost all perforations were healed within 14 days. Small sized (0.2 mm) perforations did not show faster healing as expected rather healing was slower compared to other two groups.

4.4.3 Making a chronic perforation model

Daily instillation of 1% hydrocortisone was applied around the perforation on 4 groups of rats. First group received only one instillation of hydrocortisone and another three groups received daily instillation for 3, 7 and 10 days respectively. At the end of last group's cortisone instillation, otomicroscopic examination was performed at every alternate day for 4 weeks. At the end of 4 weeks 1 day and 10 days instillation groups showed 100% open perforation but 3 and 7 days groups showed 67% and 50% open perforation respectively. As the number of animals of studied groups was small, including more animals to the group would give better conclusion, however, this small group study provided the strong indication for further evaluation.

4.4.4 Chronic TM perforations with MSC transplantation

The perforations were large to subtotal with presence of scars at the time of MSC transplantation. At 8 weeks after the transplantation, the prograf treated group showed 4 closed perforations of 5 compared to 1 of 5 in control ears. The group without prograf showed 1 closed perforation of 5 in treated ear compared to 0 of 5 in control group. One common noticeable finding for both MSC treated group was presence of amorphous materials, which virtually filled the whole middle ear cavity. Light microscopic section of a treated ear displayed pushed away lamina propria towards the external auditory canal wall by amorphous material of middle ear cavity. Electron microscopy showed a well structured lamina propria like in the normal TM in-between the islands of tissue.

5 DISCUSSION

A chronic TM perforation is a significant clinical problem in the field of otolaryngology. The only available treatment is the surgical repair and most common surgical practice is repair with autologus transplants of temporalis fascia, perichondrium or cartilage. There are no exact available statistics about how many people that are suffering from this problem around the world, but it is a big problem especially for the underdeveloped and developing countries. Due to lack of adequate treatment facilities and resources only a tiny group of people from the affluent society can afford this costly treatment. Millions of people are bearing all the consequences of chronic TM perforation throughout their life and are left behind from this surgical treatment. Researchers have been working for several decades to find-out a simple office technique to replace the conventional surgery so that the millions of less fortunate patients can be attended. Any attempts to find an alternative, easily delivering technique for wound repair demands better understanding of the biology of wound healing in the TM.

5.1 THE TM HEALING

The healing of a TM perforation differ in the reparative processes to that of other cutaneous structures (Reijnen and Kuijpers, 1971). Epithelial proliferation starts to the close vicinity of the perforation and migrates towards the perforation to span the gap. Progression of proliferating epithelial from the two opposite side of the perforation is halted when they meet each other, by contact inhibition, so the gap seems to be fully covered. Now the perforation looks apparently closed without any wound strength. The remodeling continues by invasion of granulation tissue beneath the epithelial layer. A significant number of studies have been performed using different substances (Laurent et al., 1991; Spiegel and Kessler, 2005) for healing TM perforations. The ultimate goal for each and every study is to get a healed TM which behaves and functions similarly with the normal TM.

5.2 CHRONIC PERFORATION AND PERFORATION SIZE

An acute perforation heals spontaneously in both animals and humans so it cannot serve as a model analogous to a long-standing, chronic perforation situation which is commonly experienced in man. It is essential to test the efficacy and probable adverse effects of any newly developed drug or treatment in the animal model prior to human clinical trials. Only few animal models with chronic TM perforations have been reported (Amoils et al., 1992; Kaftan et al., 2004). In most of these studies the TM perforations were performed by using a myringotomy lancet, surgical knife or any other sharp cutting surgical instruments. In all of our studies a KTP laser beam with different diameter of laser fibers were used to create the perforation. The advantage of using laser is the selection of a precise location on the TM and it's highly reproducibility. One possible disadvantage is the small size of the perforation depending on the limited fiber diameter. This problem can be overcome by giving repeated shots to enlarge the perforation. Hydrocortisone (Spandow and Hellstrom, 1993), mytomycin C (O'Reilly et al., 2001), gluteraldehyde (Truy et al., 1995) and some other chemical substances have been used for creating a chronic perforation model. We have adopted the hydrocortisone protocol (Paper IV) due to its efficacy, availability and cost effectiveness. Hydrocortisone prevents inflammation and thus blocks the first step of the healing process.

In general, the size of the perforation was thought to have an impact on the healing time, although there is a lack of documented reports in this regard. One study designed with different perforation sizes running from 1mm to three-quarters of the pars tensa diameter reported no correlation between the healing time and size of the perforation (Wang et al., 2004). Our findings (Paper IV) also support the above evidence where the smallest size (0.2 mm) perforation did not heal faster than that of the three times larger perforation.

5.3 MEASURING THE STRENGTH OF THE TM

The normal architecture and the physical properties of the TM are important in the hearing pathway. There are very few reports on the mechanical properties of healed and reconstructed TMs. To the best of our knowledge, a number of normal TMs and a few minor perforated gerbil's TMs were measured with MI where the highest applied pressure was 200 daPa (von Unge et al., 1993). We have increased the pressure up to 350 daPa and tested both the normal and recently closed, myringotomized TMs at two and four weeks post operatively (paper I) and in long-term measurements at six months after myringotomy (paper II) in the rat model. The anatomy and physical behavior of the rat TM closely resembles that of the human, so we adopted the rat model for testing the stiffness and deformation against the applied pressure. A mouse model has also been adjusted to the MI set-up, but the smaller size of the TM and higher pressure made the effort unsuccessful in more than half of the preparations. In clinical practice it is known that the middle ear pressures and TM pathologies are closely connected but the underlying cause is not known yet. Lots of clinical questions concerning the behavior and functionality of the TM with regards to static ME pressures is still unresolved. In general, tympanometry is used to study the behavior of the TM where acoustic impedance of the ME is measured while applying pressure to the external auditory canal (von Unge et al., 1991). This technique is simple but only gives information about the overall compliance of the TM. High accuracy measurements of amplitude and phase of the cat vibrating TM has been reported by using a heterodyning interferometer (Decraemer et al., 1989). Apart from the static behavior, response to the sound waves is also an important factor in the restoration of a functional TM. Laser Doppler interferometers allows to measure the motion of the vibrating TM (Vlaming et al., 1984) and it's incorporation with computer-controlled apparatuses makes it convenient and it is being widely used in clinical research set-ups (Stasche et al., 1994). The advantage of MI method is that, it provides a full-field, 3-dimensional, overall stiffness change along with local stiffness variation of different portions of the TM.

Applying this MI method in our rat models, already at two weeks post-myringotomy showed an almost normal strength (paper-I) and at six month it was slightly reduced (paper-II). We now know from these studies that a rat TM withstands the same pressure after two weeks post-myringotomy as a normal unperforated TM. If we translate that in the human clinical situation, the time for facing the pressure such as diving and flying after healing might be reduced.

5.4 HISTOLOGICAL STUDIES

Histological sections revealed a five-fold increased thickness of the lamina propria as compared to the control TMs at two and four weeks post myringotomy. Excessive proliferation of fibroblasts and extra-cellular substances with a production of fiber-like structures with bizarre orientation was observed in the short-term healing group. The underlying mechanism of this excessive production might be to restore the strength to the normal TM in the earliest possible time. The mean fiber count of myringotomized and healed TM at six months was significantly higher than that of two and four weeks. These fiber counts were performed based on TEM sections, so they can not be judged as an absolute count but rather than a relative reflection. In a short-term study the site of the perforation could be detected and the architecture of the lamina propria close to the perforation was quite normal. In the long-term study the sharply cut edge of the lamina propria could not be identified, probably due to the integration of the original lamina propria to the newly proliferated fibers and extra-cellular substances. The cell contents and the number of vessels appeared to be diminished in the long-term group as compared to the short-term group. It indicates that the biological activities still continues but somewhat decreased in the later five months as compared to the results in the short-term study. Whether this stage of repair is the final stage or if the reparative process will further continue is not yet explained. It is not reported yet whether the healed TM will ever return to it's original architecture after myringotomy. The presence of scar tissues in the lamina propria in the long-term group obviously fulfill at least one function, to keep the TM free from perforation, a prerequisite in restoring the normal gas composition within the middle ear cleft and thereby reshaping a normal environment for biological mechanisms in the ME. A striking finding in the histological study of the myringotomized and closed TMs was that all the stem cell treated ones showed a larger increase of thickness as compared with the untreated control TMs. We speculate that growth factors and other secreted substances from the transplanted stem cells might influence the proliferation of fibers and ground substances although these parameters were not tested in this study. It was possible to presently describe in detail the exact closing process after myringotomy in acute and chronic perforations which is of importance for the clinical situation. It is now known that the repair includes a significant thickening of the TM due to an increase in the ground substances and collagen fibers even though the TM looks normal in clinical inspection.

5.5 STEM CELL TRANSPLANTATION AND PERFORATION

Recent advances in developmental biology and tissue engineering makes it possible to test the efficacy of cells supplied from exogenous sources, and mobilizing the cells from endogenous origin for repairing damaged or lost tissues. ES cells are in the center of interest because these cells can be pre-committed towards a specific cell lineage and they can complete their maturation in an *in-vivo* situation. A recent study reported quite convincing evidence that human ES cells can electrically integrate in the recipient myocardium and thereby contribute to the augmentation of the pumping action following an ischemic injury (Kehat et al., 2004). Another study reported the enhanced healing of acute TM perforation by using mouse ES cells on gerbils TM (von Unge et al., 2003). In our study (Paper III), we did, however, not find any enhanced healing of acute perforation after using mouse ES cells on the myringotomized rat TM. There was, however, significant thickening of the treated TMs as compared to the controls. This

thickening of the TM does not interfere with the displacement or stiffness of the myringotomized and healed TM. This is of great benefit for the clinical patients to have an augmented repair of a perforated TM. Whether these structural changes will influence the patients hearing has not been addressed. Another positive finding was the absence of teratoma formation after the considerable long period of six months after treatment. Shortage of donor organs for clinical transplantation and increasing needs for available organs has focused attention on the possibility of xeno-transplantation. One clinical study reported that the transplanted porcine fetal pancreatic cell produce insulin for a limited time after transplantation to diabetic patients (Groth et al., 1994). It is not clear yet how different more or less closely related species accept xeno-transplantation.

The large range of plasticity and the ability of bone marrow derived MSC to secrete growth factors for tissue repair promotion influenced us to investigate the effects of these cells on wound healing. Improvement of healing was reported both in fascial and cutaneous incisional wounds by using MSC (McFarlin et al., 2006). It was demonstrated that the acceleration of the wound healing was not restricted to a particular type of wound. They also reported the increase in the levels of collagen fibers in fascial wounds which is crucially important for the tensile strength of the treated wound. Our findings (Paper IV) on the histological study also reported an excessive increase of ground substances and fibers in MSC treated TMs, although we did not perform any quantitative measure of the amount of collagen fibers. A striking finding in this study was a high rate of perforation closures in the MSC treated ears as compared with the control.

Graft rejection by the recipient's immune system is a major concern in this field of transplantation therapy. Scientists are constantly making efforts to overcome this hurdle. Significant achievement has been reported in experimental protocols using small animal models in generating graft accommodation and donor specific tolerance. In case of accommodation, the antibody production in transplanted animals was delayed, and when the antibodies were allowed to return later, the transplanted organ had developed means of protection from these antibodies, thus preventing antibody-mediated rejection (Lin et al., 2000). In tolerance studies, the immune system of the recipient was manipulated so that it learned to recognize the foreign graft as "self" (Galili, 2004). Introduction of immuno-suppressive agents did not show any improvement in healing when treating acute TM perforation with ES cells (Paper III). On the other hand, when treating a chronic perforation, however, the highest number of perforation closures was recorded in the immuno-suppressed group (Paper IV). We speculate that the chance of an immune rejection is bigger in highly vascularized organs than the least vascular ones such as the pars tensa. Therefore the TM might be a suitable organ for cell transplantation, in this regard.

6 CONCLUSIONS

This study is an accumulative effort and comprises a series of experiments to find a simple office treatment for chronic TM perforations, which is a long-desired goal for the otolaryngologist. It was an attempt to describe and define the pertinent features of the acute and chronic perforation and their physical and structural characteristics after the healing, and possible effects of stem cell treatments on the perforated TM. In cutaneous wound healing the tensile strength of the healed wound at two weeks post-trauma is minimal and the increase in strength starts rapidly in the remodeling phase. Our findings in the short-term study indicate that the stiffness and strength of the myringotomized and closed TM regain the strength close to normal already at two weeks post-myringotomy.

In the histological study at two and four weeks post myringotomy an intense proliferation of extracellular substances and collagen fibers were observed. The fibrous architecture of the lamina propria close to the perforation was maintained in the short-term study. Histological analysis at six months post-myringotomy revealed a disorganized lamina propria with bizarrely oriented collagen fibers and the presence of fibroblast, vessels, extracellular substances and some edema. These findings imply that the healing process is not yet completed, but still progressing at a slower pace in order to reshape the structures towards the normal.

The use of laser for creating the perforation makes it possible to standardize the size and reproducibility. The size of the perforation did not have any effect on the healing time. On evaluating the previously performed chronic perforation model by using hydrocortisone, our study gave an insight for further research with the minimum application of hydrocortisone for creating chronic perforation. Transplantation of mouse ES cells in the acute perforation did not show any augmented healing. No evidence of teratoma formation, known as a complication of ES cell treatment was detected after the relatively long period of six months. ES cell treated ears revealed a thickened TM in the histological study which suggest that stem cells might secrete growth factors or other substances which stimulate the production of connective tissue and extracellular substances.

Transplantation of human MSC in the chronic perforation exhibited better healing rate as compared with the untreated controls. The histological study showed a huge proliferation of granulation tissue in all MSC treated ears which almost completely filled the middle ear cavity. Administration of an immunosuppressive drug delivered mixed results and it demands further evaluation. The results of the animal study give some important information about the basic reparative process of TM healing which can be incorporated for further clinical understanding. Based upon the above findings, we conclude that this is the very beginning of the exploration of stem cell treatment for TM perforations and that it keeps a promise for future improvement and modification in this respect.

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