Outlook for tissue engineering of the tympanic membrane

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Abstract

Tympanic membrane perforation is a common problem leading to hearing loss. Despite the autoregenerative activity of the eardrum, chronic perforations require surgery using different materials, from autologous tissue - fascia, cartilage, fat or perichondrium - to paper patch. However, both, surgical procedures (myringoplasty or tympanoplasty) and the materials employed, have a number of limitations. Therefore, the advances in this field are incorporating the principles of tissue engineering, which includes the use of scaffolds, biomolecules and cells. This discipline allows the development of new biocompatible materials that reproduce the structure and mechanical properties of the native tympanic membrane, while it seeks to implement new therapeutic approaches that can be performed in an outpatient setting. Moreover, the creation of an artificial tympanic membrane commercially available would reduce the duration of the surgery and costs. The present review analyzes the current treatment of tympanic perforations and examines the techniques of tissue engineering, either to develop bioartificial constructs, or for tympanic regeneration by using different scaffold materials, bioactive molecules and cells. Finally, it considers the aspects regarding the design of scaffolds, release of biomolecules and use of cells that must be taken into account in the tissue engineering of the eardrum. The possibility of developing new biomaterials, as well as constructs commercially available, makes tissue engineering a discipline with great potential, capable of overcoming the drawbacks of current surgical procedures.

Introduction

The hearing process consists on the transformation of mechanical energy of sound waves in a biochemical signal that stimulates specific receptors, which trigger a nerve impulse. This process starts by the transmission of the acoustic pressure at the tympanic membrane (TM). This membrane separates the outer ear from the middle ear and plays a crucial role in sound perception and in the protection of the medium ear. Vibrations of the TM are transmitted and amplified through a chain of mobile ossicles. Finally, the movement of the inner ear fluids stimulates the mechanoreceptors at the hair cells of the cochlea, allowing the perception of sound.

The TM has a trilaminar structure, with an outer layer of stratified squamous epithelium composed by keratinocytes; a middle layer consisting of fibroblasts and type II and type III collagen (lamina propria), whose function is to provide mechanical strength, consistency and elasticity; and an inner non-keratinized mucosal epithelium (Figure 1). The eardrum consists of two parts: pars tensa (PT), where majority of perforations occur, and pars flaccida (PF), and the main difference between them is the composition of the lamina propria, which is perfectly adapted to the specific role of the TM.

Thus, the lamina propria of the PT consists mainly of type III collagen in the inner layer (but also of type II and I), while the external layer consists predominantly of type II collagen and, to a lesser degree, type III and I collagen. Furthermore, the lamina propria of the PF is constituted by loose connective tissue and a few elastic fibrils. The eardrum’s shape is oval, and the collagen fibrils are arranged in a radial and circular direction in the outer and inner layer, respectively. Despite the current data on the TM thickness being limited, it is commonly accepted that it is not homogenous, varying from 30 to 150 µm, and with a mean value of 74 µm. The functionality of the TM depends upon its specific structure. Therefore, if the TM is altered (such as in a tympanic perforation), a transmission hearing loss may occur.

TM perforations are a common problem. The most frequent causes are middle ear infections, traumatic rupture caused by an increased pressure and postoperative complications. These injuries can cause hearing loss and recurrent infections. The perforations of the TM can be classified according to their duration, in acute and chronic (more than three months), and by the presence or absence of drainage, in wet and dry perforations. The time of healing and rate of TM perforations closure strongly depends on the type of perforation. Thus, acute and wet perforations close spontaneously after a few weeks in about 76-94% of cases, by using only a topical antibiotic therapy. However, the closure of chronic perforations needs surgical intervention (myringoplasty) to restore the function of the membrane.

The closure of TM perforations has been investigated for centuries, in order not only to avoid hearing loss, but also to prevent recurrent infections. The first approaches to repair the TM date from 1640,
when Banzer used pig’s bladder to cover these injuries. In 1848, Yearsley and Toynbee proposed two artificial TMs consisting of a cotton pellet hydrated with glycerol and a disc of natural rubber, respectively. Previously, many authors had used a number of materials to restore tympanic perforations and several patents of TM were issued, but the first TMs were just instruments with a protective function, rather than with a regenerative capacity, and they were unable to improve hearing. However, these early works laid the background for the development of a surgical treatment of tympanic perforations.

Berthold performed the first myringoplasty in 1878, in which he achieved the complete closure of the TM by using a skin graft. In the 1950s, Hagerman and Ortegen introduced the autologous temporal fascia for myringoplasty, and this graft became the most used in otologic surgery.

Currently there are two therapeutic procedures - myringoplasty and tympanoplasty - to close TM perforations and restore the TM integrity and function. Although both techniques have a high success rate, both of them require surgery under general or local anesthesia and an incision to obtain the graft material. Moreover, surgery complications can result and multiple interventions are sometimes required to achieve a complete closure of the perforation.

Therefore, there is a current need of cost-effective, non-surgical and safer alternatives. So, tissue engineering focuses on three basic principles to restore the anatomy and functionality of the TM: scaffolds made from various materials, growth factors and cells. However, the studies conducted so far are inconclusive and they have proven their efficacy only in animal models or in reduced groups of patients. Nevertheless, tissue engineering is a new and promising interdisciplinary field that can overcome the drawbacks and limitations of current surgical techniques.

This systematic review aims to analyze the regeneration of the TM by using tissue engineering as an alternative to conventional surgical procedures. It will focus on the use of different scaffold materials, cells types and growth factors.

Materials and Methods

The sources of information used to develop this systematic review were:
- A search of the online database of the National Library of Medicine (PubMed) performed from January to June 2014. The key terms tympanic membrane repair, tissue engineering of the tympanic membrane, tympanic membrane regeneration were searched. The abstracts of the previous search were examined and included for the current review according to their usefulness to the topic being reviewed. Date of publication, presence of experimental data and the reference to the use of scaffolds, growth factors and/or cells were considered.
- Hand search of the reference lists of these articles was performed. The total articles included for full review was 79. Of those, 33 corresponded to experimental studies performed in animals, 21 were carried out in humans and 8 were in vitro studies. Among the included articles, 35 corresponded to studies of scaffolds, 32 to biomolecules research and 9 to studies with cells. Some works, such as reviews or patents, were not classified in any of these categories.
- The origin of the included articles was as follows: Europe (15), USA (14), Japan (8), Korea (8), Turkey (7), China (6), Australia (3), Canada (2), Brazil (1) and India (1). Some works, such as reviews or patents, were not included in this distribution.
- Personal contacts with specialists of tissue engineering of the TM.
- Specialized textbooks of ear histology and otology for the management of tympanic perforations.

Current treatment of tympanic membrane perforations

Most of TM perforations close spontaneously in few weeks and require only a treatment to prevent water from entering the ear and infections. However, if the lesion does persist, a surgical intervention is needed.
After an eardrum injury, an exudate composed by lymph, interstitial fluid and/or blood clots is secreted around the edges of the perforation. This secretion forms a layer that protects the underlying tissue from dehydration and provides a support for cell migration. Within days, the proliferation of the squamous epithelial layer occurs and the produced keratin migrates towards the centre of the perforation. Finally, the layer of connective tissue closes the perforation.19

Thus, TM repair occurs mainly by epithelial migration and the function of the different materials used is to act as scaffolds to guide the cell migration from the edges of the perforation. Nowadays there are two clinical treatments to repair the eardrum: myringoplasty (also known as type I tympanoplasty) and tympanoplasty. Both procedures use a material situated in the tympanic cavity, under the perforation, whose function is to act as a support for the regeneration of the TM. The main difference between these techniques is that tympanoplasty usually involves the repair not only of the TM, but also of the mobile ossicles that transmit sound from the eardrum to the inner ear. Tympanoplasty is also the surgery used in large or recurrent perforations. Both techniques use resorbable materials, such as Spongostan® or Gelita® and patient’s own tissue.7 In both cases, the surgical procedure consists in replacing the perforated eardrum by an artificial one, facilitating the perforation closure by providing a patch on which the neomembrane grows.20

Myringoplasty consists of the debridement of the perforation’s edge to provide cells and on the use of a graft that is placed under the remnants of the TM. This graft is inserted through a retroauricular, endoaural or endomeatal incision and it is supported with a resorbable material.21 The graft can be placed under the margins of the perforation with a small part extending over the posterior canal wall (underlay technique) or upon the outer surface of the tympanic membrane, with a slit to tuck it under the handle of the malleus (overlay technique). The 10-year graft success rate is around 80%, with a recurrence rate for chronic otitis media of 15% and 26%, using overlay or underlay technique.22 There are several materials currently used in the clinic, such as perichondrium, vein, cartilage - from the concha or tragus -, fascia and fat, and numerous studies have been conducted to assess their efficacy.16,21,23 Thus, the cartilage-perichondrium graft has proved to produce better results than temporal fascia and perichondrium of the tragus, regarding hearing improvement and TM morphology.24

Despite the wide range of available grafts, the temporal fascia remains the gold standard in the clinical practice.2 However, this graft has some disadvantages such as infection and autolysis. Therefore, the usefulness of other materials for TM closure has been extensively investigated.17,25 The autologous fat is one of the grafts on which most attention has been focused in recent years. By using this material, the rate of success in TM closure ranges between 76-92%, while this rate is 83% in paper myringoplasty.16,17 The adipose tissue graft has the advantages that it is easily and quickly obtained with a low morbidity and it also has a rate of success similar to that of the temporal fascia.25 Additionally, fat grafts secrete angiogenic and growth factors that promote neoavascularization and tissue repair, thus increasing the poor blood supply around the TM perforation.17,25

Normally, the fat graft is limited because it is extracted from the earlobe or post-auricular subcutaneous tissue. Therefore, alternative sources of fat tissue have been explored, such as abdominal fat, which has been used in maxillofacial plastic surgery.16 The umbilical region allows the extraction of large amounts of fat tissue without cosmetic problems, which are common in areas where the fat supply is poor or there is scar tissue from previous injuries.16

Furthermore, adipocytes from the abdominal fat are less compact and have less fibrous tissue than the earlobe adipocytes, thus promoting angiogenesis.16 Therefore, abdominal fat is a safe and effective alternative for the closure of the TM.

The selection of the ideal material for TM repair is still under research and there are many types of grafts successfully used in various myringoplasty approaches. Even if temporal fascia or periostiondrium are the most frequent choice, adipose tissue grafts have been used for the repair of small eardrum injuries with similar success rate to temporal fascia.25

Despite the large variety of scaffolds and surgical techniques available, there is still no consensus on the optimal alternative for the TM repair and the current techniques have numerous limitations (Table 1).1,2,21 As a result, there is a current need of new therapeutic approaches that overcome these drawbacks. Tissue engineering is an alterna-

| Advantages | Myringoplasty/Tympanoplasty |
|------------|----------------------------|
| High success rate | Required anesthesia |
| Good outcome in small perforations | Greater surgery time |
| Minimally invasive technique | Open surgical procedure (associated risks) |
| Routine clinical practice | Incision to take the graft and remove squamous epithelium |
| | Limited availability of autologous graft in revision cases |
| | Failure of perforation closure due to the deficient |
| | regenerative activity at the edges of the injury |
| | Frequent re-perforation |
| | Bilaminar neomembrane: flaccid and acoustically suboptimal |
| | Side effects: retraction pockets, tympanosclerotic mass, rejection |

| Disadvantages |
|---------------|
| Surgery simplification | Mostly animal studies (acute perforations, which would spontaneously close in most cases) |
| Cost savings | Lack of a standard animal model |
| Improve outcome in chronic perforations | Scarce human clinical trials |
| Growth factors improve tympanic closure | Possible side effects of scaffold materials, biomolecules and cells |
| Specific design of scaffold materials that reproduce the mechanical properties of the eardrum | Ethical and legal issues concerning the use of xenografts |
| Possibility of generating a commercially available tympanic membrane | Complex manufacture of the artificial construct (storage, biopreservatives, quality controls, production and transportation costs) |

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Table 1. Comparison between current surgical techniques and tissue engineering for the regeneration of the tympanic membrane.
Tissue engineering of the tympanic membrane

There are three main problems to achieve the repair of TM perforations: i) the lack of a structural support; ii) the scarce angiogenesis and growth factors; and iii) a deficient extracellular matrix leading to a weak cell adhesion in the neomembrane.2

Tissue engineering has been applied by two approaches: in vitro - constructs generated in tissue cultures or bioreactors -, and in vivo. This discipline employs three main elements: cells, scaffold materials - which serve as a mechanical support for cell proliferation, migration and differentiation - and biomolecules - which provide a suitable biochemical microenvironment.2,21,26 TM perforations can be repaired in three ways: i) creating an artificial TM by combining a scaffold material, biomolecules and cells, and applying this membrane to the perforation; ii) applying a scaffold to the perforation and then supplying biomolecules by drops; and iii) placing a scaffold previously moistened with bioactive molecules.2 Tissue engineering methods for TM regeneration have been investigated for a few years, as several authors have utilized scaffolds with exogenous biomolecules in order to repair tympanic perforations. Nevertheless, nowadays this term is being used to refer to the design of biomaterials with different size and shapes. This review will consider separately the three elements of tissue engineering (scaffold materials, biomolecules and cells), although these three factors can be applied alone or in combination.2,7

Scaffold materials

The TM has an intrinsic regenerative capacity. However, the repair process of this structure differs from other tissues, because there is not a support under the epithelial layer. For this reason, the current clinical procedures use different scaffolds or grafts that act as a support to facilitate cells and nutrients migration towards the perforation.19

The most used grafts are fascia (temporal, lata or muscle), fat, cartilage and perichondrium, but tissue engineering is focusing on the research of a large number of novel materials.2,15,37,37 The ideal scaffold should have certain characteristics, but a material that meets all these criteria does not exist yet. Therefore, the new discipline of tissue engineering aims to improve the properties of the current scaffolds to achieve most suitable materials and better results in the TM closure.2

Scaffold materials are generally classified into decellularized tissue and polymers.2

Decellularized tissue

The decellularized tissue is obtained after cell removal of allografts or xenografts. It preserves most of the biological and mechanical properties of the original extracellular matrix. Thus, this type of scaffolds serve as templates for reconstruction and tissue remodeling, as they retain the native structure and contain functional proteins, such as collagen and proteoglycans.2,7,28

Some of the materials that have been studied are: acellular collagen from cadavers or porcine peritoneum,29,30 AlloDerm® - a cryo-dried acellular dermal tissue matrix from human donors -2,7 Strattice® - obtained from porcine skin -31 decellularized urinary bladder, SurgiSite® - porcine small intestinal submucosa -2 and Tutopatch® and Audiomesh®. Tutopatch® and Audiomesh®. Moreover, the human application of decellularized tissue is difficult, due to ethical and biosafety issues, as it derives from tissue of cadavers or other species.

Polymers

Polymers have many advantages over the above scaffolds, mainly because their shape, size and porosity can be changed with great ease to better fit their future application. Furthermore, they are biocompatible, biodegradable, easy to synthesize and handle, can be produced on a large-scale basis and their degradation and mechanical properties can be controlled. Nonetheless, polymers may not be able to reproduce the native structure of the membrane. Some of the polymers investigated to achieve the repair of the TM are listed in Table 2.2,18,22,22,31-46

Gelfoam® is a gelatinous sponge obtained from denatured swine skin. It has been used as a scaffold for tympanic and ossicular grafts and in traumatic perforations.2,22 Another researched material is polyllysine polymerized with latex from Hevea brasiliensis tree, which induce a greater vascularization when externally applied to a fascia graft.23

Silk fibroin is also a useful support for TM regeneration. It is obtained after the removal of the antigenic component - sericin - of Bombyx mori silk and presents numerous advantages, such as elasticity, biodegradability and biocompatibility. In fact, it has been used for years as a suture material. It also presents ease to bind peptides, thus facilitating cell adhesion, and it can be processed in a large number of structures (fibers and sponges).2,34,35 Some studies have shown that human keratinocytes from the TM have a better growth in silk patches than in other scaffolds. These results are supported by in vivo studies in which, by using a support made from silk fibroin, tympanic perforation closure was achieved in less time and in a more organized way and with a faster hearing improvement.7,30,35,36 An artificial TM whose main component is silk fibroin has recently been patented.20

Chitosan is a N-deacetylated form of chitin, which is a mucopolysaccharide with anti-bacterial properties present in the exoskeleton of arthropods. Chitosan promotes healing of skin, bone and liver wounds and has been successfully tested as water-soluble and water-insoluble patches. Three-dimensional chitosan patch proved to have advantages with respect to other support materials.7,18,37,38

Alginate, a natural polymer derived from seaweed, serves as support to the growth of mucosa and keratinized epithelium. It is also used as a delivery system for tissue regeneration, although further research is needed to assess human safety and efficacy in the long term.2,7,39

Moreover, hyaluronic acid, which can be used as a solid scaffold (with different chemical modifications) or as a topical biomolecule, has shown its usefulness in tympanic repair.2,40-43

Synthetic materials, such as polyglycerol sebacate (PGS), have also been tested. PGS is a biodegradable polymer obtained by polycondensation of glycerol and sebacic acid that has many advantages.2,7,44 Hydrogels and sponges derived from glycosaminoglycans could be also useful in tissue engineering of the TM. Among them, Carbylan-GSX promoted TM closure in less time and without inflammatory reaction.7,47

Finally, a bilaminar atelocollagen-silicone membrane soaked with fibroblast growth factor is one of the most recent and promising inventions, which will be explained later.

Biomolecules

Growth factors stimulate wound healing in many tissues. These biomolecules and their receptors are expressed during the regeneration process of the TM, as shown in animal trials.3,7 Perforation closure occurs by epithelial proliferation and migration. Therefore, molecules, such as exogenous growth factors, that enhance regenerative processes could help to close TM perforations.19,48

There are five main groups of growth factors: epithelial growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor...
Table 2. Polymers used for tympanic membrane regeneration by tissue engineering.

| Polymer                              | Model          | Properties and main findings                                                                                                                                                                                                 |
|--------------------------------------|----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Gelfoam®                             | Human          | Hemostatic absorbable material used to hold the graft and as a scaffold to growth factors delivery Biocompatibility Faster tympanic closure Increased tympanic closure rate                                           |
| Polylsine polymerized with latex     | Human          | Induction of greater tissue vascularization (possible presence of vascular growth factor, which improves vascularization of the fascia graft by promoting angiogenesis) Biocompatibility (no toxicity or allergic reaction) No significant changes in hearing function or rate of healing |
| Silk fibroin29,33-35                  | In vitro       | Maintenance of keratinocyte growth and cell adhesion and integrity Facilitation of continuous epithelium growth without deforming the membranous contour Supplying continuously a hydrated surface |
| Silk fibroin29,33-35                  | Animal         | Transparent and trilaminar structure of the neomembrane similar to the native membrane More organized growth of epithelial cells and connective tissue Faster tympanic closure and hearing recovery |
| Chitosan18,36,37                      | Animal         | Healing rate comparable to natural healing rate Higher collagen density and more organized structure than in natural healing Better proliferation of tympanic membrane cells Biocompatibility Better quality neomembrane than that obtained with paper patches Good cell proliferation Porous structure that ease cell infiltration Trilaminar structure of the neomembrane similar to the native membrane |
| Calcium alginate                      | Animal         | Growth promotion of mucosal and keratinized epithelium Cross-linking with calcium facilitates its manipulation Greater rate of tympanic closure than with paper patches Possible human risk of ototoxicity |
| Hyaluronic acid29-42                  | Animal         | Reduced time and increased rate of tympanic closure Better quality of the neomembrane Its application on its own (Epifilm®) does not improve perforation closure rate |
| Poly (glycerol sebacate)             | Animal         | Epidisc™ application with a fat graft showed a closure rate similar to fat or cartilage myringoplasty and reduced surgery time Biodegradability Promotion of neovascularization Suitable histological structure of the neomembrane Need to accelerate degradation rate to promote optimal regeneration of the tympanic membrane |
| Hydrogels and sponges derived from glycosaminoglycans | Animal         | Increased re-epithelialization Reduced time of tympanic closure (Carbylan-GSX) Variable results depending on the specific type of hydrogel Possible inflammatory reaction (CS-SX) |
| Bilaminar atelocollagen- silicone membrane | Human         | Biocompatibility Silicone retains collagen configuration and dampness Its application with bFGF improves the results of tympanic closure (success rate, time and hearing recovery) Often need to repeat the procedure to achieve complete perforation closure |
Table 3. Biomolecules used for tympanic membrane regeneration by tissue engineering.

| Biomolecule | Properties | Model | Main findings |
|-------------|------------|-------|---------------|
| EGF         | Mitogenic effect | Animal | Greater tympanic closure in less time |
|             |            |       | Neomembrane thickness and histology similar to the native membrane; the thickness achieved in spontaneous closure is less than half the normal thickness of the TM |
|             |            |       | Stimulated neovascularization and fibroblast number |
|             |            |       | Stimulated proliferation (mainly of the squamous layer) |
|             |            |       | Long-term application leads to re-perforation and cholesteatoma formation |
| TGF-α       | More effective than EGF in promoting colony dispersion and injuries healing | Animal | TGF-α was not observed in normal TM, but it was expressed after perforation |
|             |            |       | Greater pro-motility activity than EGF |
| TGF-β       | Chemotaxis induction | Animal | Reduction of perforation closure time |
|             | Extracellular matrix production | | Need to repeated application to achieve the above beneficial effects |
|             | Angiogenesis stimulation | | Possible formation of a disorganized fibrous scar |
|             | Possible excess of scar tissue |
|             | Thicker TM |
| FGF         | Stimulation of fibroblast, endothelial cells and keratinocytes proliferation and differentiation | Animal | Epithelial and/or connective tissue hyperplasia |
|             | Increased success of tympanic closure when applied directly to the perforation; Stimulation of collagen fibrils growth |
|             | Vasodilation promoting |
|             | Stimulation of protease production |
|             | Human | | Increased tympanic closure rate and reduced time of TM closure |
|             | Enhanced hearing recovery |
|             | Possible epithelial pearl formation |
|             | Reduction of middle ear infections |
|             | Hyperplasia of granulation tissue - which disappears in 5-7 days |
| KGF         | Reactive oxygen species detoxification | Animal | Enhanced epithelial migration and proliferation in the first steps |
|             | Promotion of re-epithelialization |
|             | Keratinocytes proliferation and migration |
| PDGF        | Fibroblast mitogen | Animal | Increased tympanic closure rate |
|             | Reduction of perforation closure time |
|             | More abundant connective fibrous tissue layer |
|             | Human | | No increase in tympanic closure |
| VEGF        | Fibroblast mitogen | Animal | VEGF is more specific and important than bFGF in acute perforation closure |
|             | Angiogenesis stimulation |
|             | Induction of collagen deposition |
|             | Induction of epithelialization |
| Autologous serum eardrop | Promotion of wound healing | Human | ASET does not require anesthesia, reduces or completely closes chronic perforations and implies a continuous supply of growth factors by the own patient |
|             | Lack of antigenicity |
|             | Large quantity of growth factors |

EGF, epidermic growth factor; TM, tympanic membrane; TGF-α, TGF-β, transformant growth factor type α, β; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; ASET, autologous serum eardrop therapy; NGF, nerve growth factor; IGF-1, insulin-like growth factor type I.
(PDGF), transforming growth factor β (TGF-β) and insulin-like growth factor. Regarding TM regeneration, most of the research has focused in biomolecules of the first two groups. These molecules can be applied either in drops or in a scaffold soaked with them.2,7,9

EGF stimulates the synthesis of DNA, RNA, proteins and hyaluronic acid. Moreover, there are high affinity EGF receptors in tympanic epithelial and stromal cells, and after an injury in this structure, the EGF expression is parallel to the reparative process. Although the number of EGF receptors does not increase after a perforation, their inhibition delay TM closure.2 This growth factor is naturally expressed after damage, but its topical application achieved positive results in acute and chronic perforation animal models (Table 3).19,21,46-72

TGF-α is another molecule from EGF family used in tympanic regeneration, as its expression correlates with epidermal cells proliferation in the TM.5,25,55

Within the FGF family, basic fibroblast growth factor (bFGF) has been the most investigated. This factor is produced after TM injury and it facilitates the perforation closure through various mechanisms.2 bFGF acts mainly in the epithelial layer, where there are more specific receptors for it, although these receptors are also present in the mucosal layer.56 The application of bFGF in animal models increases the success rate of tympanic closure, both in acute and chronic perforations.5,57,58 In both cases, the beneficial effect of bFGF also lies in the induction of rapid proliferation of the subepithelial connective tissue.58

On the other hand, several studies in humans showed that the application of different scaffold materials soaked with bFGF achieved a higher and faster healing rate, but these results were not significant when using only the support material. This fact suggests that it is not the scaffold itself, but its combination with growth factors what is crucial in the closure of the TM.48

One of the most recent approaches with better results in human clinical trials is the application of a bilayer membrane soaked with Trafermin® (human recombinant bFGF). This membrane is composed of atelocollagen - a low-antigenic collagen obtained from calf skin collagen treated with protease - and silicone, which prevents the membrane from its introduction in the middle ear. Collagen is an excellent material to close perforations, since it is resorbable and compatible with the surrounding tissue. Moreover, it acts as a support for fibroblast growth, which infiltrate from the perforation membrane, and it also implies a shorter surgical time.22,46

Another successful approach tested in human chronic perforations is the regenerative treatment with BFGF and a gelatin sponge, which is sealed with fibrin glue.90 In addition to the growth factors mentioned above, other factors61-66 and other biomolecules, such as autologous serum, hyaluronic acid, human insulin,66 platelet-rich plasma48 and plasminogen have been used with different efficacy.

Autologous serum contains a variety of growth factors, vitamins and immunoglobulins capable of modulating the proliferation of various tissues to promote wound healing. Its administration in drops (autologous serum eardrop therapy) is beneficial in human chronic perforations. Furthermore, it eliminates the surgery procedure of refreshing the perforation edges. However, more studies are needed to clarify if the results are due to the autologous serum, the chitin support employed or the combination of both.51

Studies with human umbilical cord serum have also been conducted, as it achieves a faster healing of corneal wounds than autologous serum. The effectiveness of serum is due to the presence of molecules capable of accelerating tissue regeneration, such as growth factors

| Biomolecule                  | Properties                                                                 | Model                                      | Result                                                                                   |
|-----------------------------|---------------------------------------------------------------------------|--------------------------------------------|------------------------------------------------------------------------------------------|
| Human umbilical cord serum  | High quantity of growth factors, free radical scavengers, vitamin A, C, E  | Animal                                     | Reduced time in tympanic perforation closure success rate                                 |
| Hyaluronic acid             | Viscoelastic properties, osmotic pressure, regulation of cell proliferation | Human                                     | No increased tympanic closure success rate                                               |
| Human insulin (IGF-1)       | Neovascularization, increased fibroblast growth rate, differentiation from perforation edges | Human                                     | Beneficial effect in perforation epithelialization                                       |
| Platelet-rich plasma (PRP)  | Reduced time in tympanic perforation closure success rate, reduced size of perforation | Animal                                     | Increased fibroblast growth rate, differentiation from the perforation edges              |
| Amniotic fluid              | Hyaluronic acid, SCGF, bFGF, free radical scavengers, cytokines            | Animal                                     | No increased tympanic closure success rate                                               |

Table 3. Continued from previous page.

| Human umbilical cord serum  | High quantity of growth factors, free radical scavengers, vitamin A, C, E  | Animal                                     | Reduced time in tympanic perforation closure success rate                                 |
|-----------------------------|---------------------------------------------------------------------------|--------------------------------------------|------------------------------------------------------------------------------------------|
| Hyaluronic acid             | Viscoelastic properties, osmotic pressure, regulation of cell proliferation | Human                                     | No increased tympanic closure success rate                                               |
| Human insulin (IGF-1)       | Neovascularization, increased fibroblast growth rate, differentiation from perforation edges | Human                                     | Beneficial effect in perforation epithelialization                                       |
| Platelet-rich plasma (PRP)  | Reduced time in tympanic perforation closure success rate, reduced size of perforation | Animal                                     | Increased fibroblast growth rate, differentiation from the perforation edges              |
| Amniotic fluid              | Hyaluronic acid, SCGF, bFGF, free radical scavengers, cytokines            | Animal                                     | No increased tympanic closure success rate                                               |

(Ref: Trafermin®: human recombinant basic fibroblast growth factor; TGF-α, transforming growth factor type α; TGF-β, transforming growth factor type β; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; AGP, α1-antichymotrypsin; EGF, epidermal growth factor; TM, tympanic membrane; NGF, nerve growth factor; IGF-1, insulin-like growth factor type 1; KGF, keratinocyte growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; AGP, α1-antichymotrypsin; EGF, epidermal growth factor; TM, tympanic membrane; NGF, nerve growth factor; IGF-1, insulin-like growth factor type 1. The information is based on an example table and should be verified with the original source.)
Table 4. Cells used for tympanic membrane regeneration in animals.

| Cells                             | Model       | Main findings                                                                 |
|-----------------------------------|-------------|-------------------------------------------------------------------------------|
| Autologous fibroblasts            | Guinea pigs | Accelerated tissue regeneration                                               |
|                                   |             | Production of new extracellular matrix                                        |
| Mouse embryonic stem cells        | Gerbil      | Greater tympanic closure success rate than in controls                        |
|                                   | Rat         | Strengthened tympanic structure (resistance of higher pressure)               |
|                                   |             | No enhanced tympanic closure with respect to controls                         |
|                                   |             | Thicker lamina propria                                                         |
|                                   |             | After 6 months:                                                               |
|                                   |             | - No significant differences in TM thickness or strength with respect to controls |
|                                   |             | - No teratoma formation                                                       |
| Human mesenchymal stem cells      | In vitro    | MSCs are able to grow and differentiate to fibroblast in the different biomaterials |
|                                   |             | Synthesis of appropriate molecules of the extracellular matrix                |
|                                   |             | Type II collagen production                                                   |
|                                   | Rat         | Greater tympanic closure success rate than in controls                        |
|                                   |             | Granulation tissue formation                                                   |
|                                   |             | Middle ear adhesions                                                          |

TM, tympanic membrane.

[EGF, FGF, TGF-β, PDGF, nerve growth factor (NGF), insulin-like growth factor type 1, vitamin A, fibronectin, antiproteases and P substance. Umbilical cord serum has a higher concentration of NGF, EGF and TGF-β than peripheral blood serum, thus it might be more useful for TM repair.69

Hyaluronic acid is a natural high molecular weight polysaccharide that regulates the recovery of the fibrous layer, as it prevents the perforation edges from dehydration.2,19,70

Human insulin has been used to the epithelialization of tympanic perforations. The beneficial effects led to a pilot study in humans, which confirmed the usefulness of insulin in the closure of this type of injury. The joint application of insulin and TGF-β obtained a faster healing than if these molecules were used separately, so the combined administration could reduce the dose of growth factors.71

Platelet-rich plasma has been tested for its ability to increase the local concentration of growth factors.7 Plasminogen, a zymogen able to degrade fibrin and extracellular matrix proteins, plays an important role in tissue remodelling. Thus, it could be an alternative to repair the TM.72,73

Nonetheless, most studies have been conducted in experimental animal models. The therapeutic potential in humans has only been assessed for EGF, hyaluronic acid, PDGF, bFGF and autologous serum, and the beneficial effects were found only for bFGF and autologous serum.2,74

**Cells**

Scaffolds, biomolecules and cells interact to achieve the regeneration of the TM. However, most studies do not employ cells, because of the natural repair capacity of the TM and the endogenous cellular source from the excoriated edges of the perforation. Thus, normally only scaffolds and active molecules that are able to recruit cells for the TM are used.7 Cells can be applied by injection or integrated within a support material. Although there are various theoretical cell sources that could be used for tympanic regeneration, only a few animal studies have been realized so far (Table 4).74-78

Autologous fibroblasts seeded on acellular swine dermis or dura mater is the most studied cell source.2,75 Stem cells (SCs) have also been studied, given their regenerative capacity. Von Unge et al. used embryonic SCs from mice with promising results, due to the differentiation and integration of these cells in the TM.77,78 Application of human mesenchymal SCs showed inconclusive results.74,79

Stem cells have the crucial feature that they differentiate depending on the signals received from their environment. Hence, the ear microenvironment may influence the maturation of SCs. The aim to repair the TM is that most of the cells become fibroblasts of the intermediate layer, whose regeneration is most difficult to achieve. Thus, SCs could be used in combination with FGF to stimulate cell differentiation into fibroblasts.

Tissue engineering is also used to obtain artificial tympanic substitutes. To this purpose, biocompatible and resorbable polymeric matrices were seeded with bone marrow-derived mesenchymal stem cells, previously differentiated in vitro into fibroblasts by the addition of growth factors. Moreover, these bioconstructs experienced mechanical stress during culture to promote fibroblast differentiation. This process increased the production of type II collagen, which is a constitutive protein of the intermediate layer of the human TM.79

However, the use of SCs is linked to safety issues (infection, rejection and tumor formation), not to mention the ethical and legal issues. Therefore, studies in this field are scarce. Additionally, human progenitor cells of the TM were found in the umbo, annulus and handle of the malleus, which suggests the possibility that there are regenerative SCs in the TM itself.69 Thus, if the remaining membrane has its own progenitor cells, it would not be necessary to incorporate external cell sources to achieve the TM repair.70 Despite the usefulness of SCs in pilot studies, this last fact and the problems of using SCs, suggest that it could be difficult the application of SCs for tympanic perforation closure or to search other cell sources, such as autologous keratinocytes.7

**Considerations for clinical application**

**Scaffold materials**

Most scaffold materials achieved favorable results in the repair of tympanic perforations. However, almost all novel materials are compared with autologous tissue (temporal fascia, tragus perichondrium or auricular or tragus cartilage), which remains the gold standard, because of the advantages of employing the patient’s own tissue. In addition, the time to collect the autologous tissue and the associated morbidity do not justify the need of a bioartificial membrane commercially available.

Therefore, novel scaffold material will become an alternative to autologous tissue, if they are available in different sizes and thicknesses that allow them to fit in the different perforations. In that sense,
computer-aid design and mold injection are being useful.\textsuperscript{2,7} However, there is a current need to optimize biomaterials that reproduce biomechanical and vibroacoustic properties of the TM.

Biomolecules

Normally, growth factors improve the healing of tympanic perforations. Nevertheless, there are still doubts concerning the release vector, type of molecule, dose and duration of the therapy to get the maximum benefit. Some studies showed that these factors should be administered until the perforation is completely closed, while others showed that one single application was enough to achieve tympanic closure. Moreover, current research aims to obtain the release of biomolecule drops in a specific area, thus facilitating the administration by the own patient. If biomolecules are applied within a scaffold, it should also be considered the loading capacity, release kinetics and binding affinity.\textsuperscript{2}

Furthermore, most of trials tested the effect of biomolecules independently, but the synergistic effect of various growth factors should be considered. Growth factors stimulate the proliferation of epidermal and connective tissue. Thus, its use is linked to epithelial pearl and cholesteatoma formation. Therefore, more long-term studies are needed to assess their efficacy and safety before their clinical application.\textsuperscript{2,7,59}

Cells

So far, the use of cells in tissue engineering of the TM is scarce and all the research has been conducted in animals. This is due to the autoregenerative capacity of the eardrum and to the fact that there is an endogenous cellular source provided by the excoriation process. Even though the application of SCs achieved a neomembrane structure similar to the native TM, their clinical use is limited due to the possible side effects, high cost and ethical and legal issues.

Furthermore, the application of cells to the constructs is difficult in a clinical setting, given the complex structure of the TM. Cell culture and seeding require strict biosafety controls and it is difficult toseed different cells in the different layers of the TM, since each cell type needs specific conditions. It is also necessary to avoid epithelial cell infiltration into the middle ear, because it could lead to iatrogenic cholesteatoma formation. So, the application of cells might not be necessary if the remnants of the TM have useful progenitor cells for the regeneration process.\textsuperscript{2,7}

The main challenge to regenerate the TM is to translate the results obtained in animal experimental models to human models. Moreover, the choice of the optimal biomolecules and scaffold materials is still not resolved, and the incorporation of cells is controversial.

Conclusions

The following key points should be considered: i) tissue engineering is an alternative for tympanic closure that combines the use of scaffolds, cells and biomolecules. It focuses on the development of novel materials that reproduce the mechanical and vibroacoustic properties of the native tympanic structure; ii) current techniques and scaffold materials used for epithelial proliferation and migration produce a bilaminar - and therefore, acoustically suboptimal - neomembrane; iii) several biomolecules with proliferative and angiogenic effects such as EGF, bFGF, insulin and serum can improve the outcome in TM perforations; iv) the development of new biomaterials and commercially available constructs for the TM makes tissue engineering a discipline with great potential in otology surgery.

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