Importance of Animal Studies in the Understanding of Otitis Media

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Introduction

According to biological data, after life on Earth has gone through 4.5 billion years of evolution, our planet has an estimated 8.7 million species, taxonomically classified under the five kingdoms of Fungi, Protista, Monera, Plantae, and Animalia [1, 2], of which 80% live in tropical forests [3]. Recently, Cavalier-Smith's group [4] added three more kingdoms to this taxonomic classification: Archaebacteria, Chromista, and Archezoa. A breakdown of the kingdoms' classification shows seven sequential levels, namely kingdom, phylum, class, order, family, genus, and species.

On top of the kingdoms' classification, Woese and Fox [5] proposed a higher level of three domains on the basis of ribosomal RNA signatures, namely Archaea, Bacteria, and Eukaryotes. Human beings, belonging to the Eukarya domain in the Animalia kingdom, have been subjected to an extensive list of infectious diseases. The UNEP [3] has estimated that about 60% of all infectious diseases known to affect humans are zoonotic, and the number of emerging diseases points to an increasing trend (e.g., coronavirus disease 2019, or COVID-19).

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Otitis media and its various forms are known to spontaneously affect humans largely through infections by bacterial, viral, or fungal agents. Although a multifaceted disease, otitis media is generally classified, on a clinical or anatomical/histopathological basis, as acute, subacute, or chronic and affects the middle ear cleft, Eustachian tube, and mastoid [6].

Humans, as a part of the vertebrate and placental mammalian class of the Animalia kingdom, share some anatomical, functional, and immunological characteristics with dogs, cats, rats, mice, chinchillas, guinea pigs, monkeys, and so on. On the other hand, in humans, the common bacteria associated with otitis media are not necessarily associated with middle ear infections in other animals; many nonhuman animal models colonized with these bacteria contract middle ear infection through a direct bullae injection or nasopharyngeal inoculation in an unnatural development of the disease. On top of that, these animals experience significantly fewer spontaneous episodes of this disease, with the exception of spontaneous or induced genetic knockout in animal models, where otitis media episodes are far too common.

Although using animal models for otitis media is subject to criticism from political and animal protection groups and to strict legislation, they are relevant sources of valid, focused, and consistent information on an all-too-common disease and have significant health, social, education, labor, and economic implications. Although the findings of these kinds of studies cannot be entirely extrapolated to humans, they do provide undeniably helpful data despite the ethical challenges, especially because such procedures may not be carried out on humans.

Legislation and Ethics in Animal Studies

Animal studies and animal experiments have long been performed in many areas, such as human medicine, dentistry, animal science, veterinary medicine, pharmacology, cosmetics, biotechnology, and agrochemistry, among others. Basic studies on anatomy, physiology, drug responses, behavior,

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and so on are used for teaching even today. Other than these, the development of surgical skills and surgical techniques, disease models, drugs and testing, drug interactions, drug tolerance levels, vaccines and testing, induced serum antibodies, and many others have made undeniable contributions to the current state of the art in many fields.

Animal studies in the context of otitis media generally use rodents as living subjects for experimentation because of their known anatomical, genomic, and functional similarities to humans. These animals are small and easy to care for and have high reproduction rates and genetic stability, making them cost-effective and easy to keep in good hygiene and health conditions, as determined by related legislation.

Legislation drawn from ethics committees, welfare acts, and local and federal rules ([7–9] and others) generally advise that any experimental study must have previous ethical approval and adequate documentation for checking, reporting and reproducibility. Animal studies should have minimal time spans to reach their objectives; no animal should be subject to simultaneous or sequential experiments; sedation or anesthesia should be used when pain is possible; and deep anesthesia is needed or recommended when they are sacrificed.

Experimental Models

Many experimental models designed for studying the various forms of otitis media and have been proposed, especially by the Otitis Media Research Group at the University of Minnesota in Minneapolis, United States, focusing on the histology, histopathology, cellular and functional dynamics, immunology, disease induction, biochemistry, and treatment of otitis media.

As a fellow, the main author Marcelo Miguel Hueb (MMH) had the opportunity to work with that group under the guidance of Marcos Goycoolea, the senior author of this book, and also at other University of Minnesota facilities, including the Otopathology Laboratory, under Michael Paparella's guidance. I become acquainted with or participated in experimental studies on the continuum of otitis media after Eustachian tube obstructions [10], acute otitis media induced by microorganisms inoculated into the middle ear [11–14], round window membrane permeability [15–17], inner ear damage caused by otitis media [18–20], the dynamics of microspheres in the middle ear mucosa of Eustachian tube–obstructed animals [21, 22], the efficacy of drug-delivery systems through absorbable membranes [23–25], experimental cholesteatoma induced by a tympanic membrane perforation with epithelial migration through a scaffold and inflammatory process [26–28], studies on cholesterol granuloma [29] and granulation tissues [30], interventions in mucoid otitis media [31], and many others by different authors.

Mimicking the infectious process in humans, some of these studies used bacteria such as *S. pneumoniae*, nontypeable *H. influenzai*, and *M. catharralis* and viruses such as influenza A and respiratory syncytial virus (RSV). As a major etiopathogenetic factor for otitis media, Eustachian tube disfunction was induced in some experiments, also mimicking what happens in humans; this can be achieved through cauterization, mechanical occlusion, ligation, radiation, microorganism inoculation, and mucosa irritation.

It generally leads to an initial stage of subepithelial edema and polymorphonuclear cell infiltration, followed by serous fluid that passively and hydrostatically occupies the middle ear space thanks to negative pressure (serous stage). Later on, chronic inflammatory cells infiltrate the subepithelial layer, and epithelial metaplasia and pseudoglandular formation ensue, along with active effusion production (mucoid stage). In these studies, latex microspheres of different sizes were shown to be phagocyted, to traverse the mucosa, or to traverse the round window membrane into the inner ear. According to these studies, infection and inflammation can be controlled through the use of antibiotics (e.g., ampicillin) delivered via biodegradable membranes.

Eventually, viral or bacterial proliferation occurs in acute cases and in cases of exacerbation in chronic disease. Epithelial breaks due to subepithelial edema or inflammation have led to granulation tissue formation—at the initial stages, they are immature, without an epithelial cover, whereas at later stages, they are mature, with an epithelial cover. Mature granulation tissues and cholesterol granulomas are associated with tissue and bone destruction, which is evident in cases of experimental cholesteatoma. Effusion and the inflammatory process have been shown to act as triggers for epithelial migration to occur through mechanical bridges (e.g., absorbable membranes and mucoid effusion), leading to cholesteatoma formation.

Evolving from this broad and fundamental knowledge, current experimental research has been focused, as it should be, on the safety and efficacy of prevention and also on different treatment alternatives for otitis media. Vaccines, probiotic use, transtympanic injections aiming to elicit topical antimicrobial effects, inflammation control, and biofilm disruption are some of the foci of ongoing studies.

In one study, chinchillas parentally immunized with *H. influenza* type b (Hib) conjugate vaccines were each challenged with an intranasal inoculation of adenovirus and were shown to transudate antibodies into the middle ear effusion [32]. These authors chose the chinchilla animal model for their studies [33] and were able to show efficacy and disease control even when using a transcutaneous route for vaccination in single or polymicrobial models [34–37]. Similar results have been achieved for efficacy and disease control in a mice model with a monovalent (e.g., histidine triad protein D, choline binding protein A, or detoxified pneumolysin) or

trivalent pneumococcal protein recombinant vaccine administered intramuscularly [38].

On another study, emerging pathogens in the microbiota of the middle ear in acute otitis media have recently been shown, and studies on its full relevance in the development of or protection against the infectious process are on the way [39]. These authors mentioned several possible probiotic actions (e.g., competition, production of molecules, stimulation of epithelial cells, enhancement of epithelial barriers, modulation of immune system), and according to their study, some of these emerging pathogens could be used for the prevention or treatment of otitis media. The experimental induction of otitis media with effusion has been achieved through intratympanic histamine injection in rats [40]; the authors of that study divided the animals into four groups, and after sacrificing them, the group that received probiotics before and after the histamine injection had the highest mucosa healing, as determined by the presence of effusion and by submucosa neutrophil leukocyte counts.

In another study, transtympanic injections of *Lactobacillus plantarum* in chinchillas have been shown not to affect the inner ear in an animal model, as measured by auditory brainstem responses evaluated before, 7–10 days after, and 28 days after injection [41]. This probiotic is known to decrease *S. aureus* and *P. aeruginosa* growth in mice wounds [42] and therefore seems a promising option for the treatment of chronic suppurative otitis media.

Other than that, even the relationship, population expansion, and infection kinetics between bacterial pathogens can be influenced when wild-type bacteria are replaced by isogenic mutant strains in the transbullar inoculation mixtures of an induced co-infection model of experimental otitis media in chinchillas using nontypeable *H. influenzae* and *M. catarrhalis* and its mutant strains [43]. Another chinchilla study using inoculations of nontypeable *H. influenza* showed that bacterial populations that were able use a phasevarion (phase-variable regulon) induced a more severe disease in this animal model, showing that controlling the bacteria's gene expression could be a promising way to control infection [44].

Maintaining vitamin D levels is also a promising way to control otitis media, as suggested by a rat model that induced otitis media via nontypeable *H. influenza* inoculation, where animals on a vitamin D–deficient diet showed greater mucosal changes, increased expressions of interleukin 6, tumor necrosis factors, and decreased interleukin 10 expression compared to nondeficient rats [45]. These studies substantiate the claim that inducing microbiota competition, gene-expression control, and adequate vitamin D levels can be powerful treatment modalities for otitis media.

Other treatment modalities aiming to control inflammation, as observed in adjuvant therapies with alpha-lipoic acid in a guinea pig model for acute otitis media [46] and surfactant protein D in a mouse model for acute otitis media induced by nontypeable *H. influenza* [47], have shown good control of inflammatory changes. This research group showed in another mouse acute otitis media model induced by nontypeable *H. influenza* inoculation that animals knocked out with surfactant protein A exhibited greater mucosal changes and higher interleukin levels when compared to wild-type mice [48]. Inflammation control was also achieved with caffeic acid phenethyl ester and thymoquinone in a rat model for otitis media with effusion [49]. These studies suggest that inflammation control plays an important role in otitis media treatment.

Also, regarding otitis media treatment models, biofilm formation caused by the most common bacterial pathogens involved in its etiopathogenesis must be taken into consideration. Microbiota competition in this scenario should also be relevant, as it is in coinfections and biofilm disruptions. A recent study using a chinchilla otitis media model has shown the clearance of *H. parainfluenza* from biofilm colonies that had already been establishe when challenged with *H. influenza* [50]. In a recent study, chinchillas immunized with a biofilm-disrupting nontypeable *H. influenza* vaccine antigen were shown to have disrupted biofilms that had previously been formed or prevented their formation from nontypeable *H. influenza* [51]. In another chinchilla model of chronic otitis media with nontypeable *H. influenza*, biofilm formation happened after middle ear inoculation; tympanostomy tubes were placed, and ofloxacin, monoclonal antibodies against DNABII bacterial proteins, or a combination of both were introduced in the middle ears [52]. The animals that received the mixture performed better against biofilm disruption and on bacterial clearance when compared to the single-drug animals.

These experimental studies suggest that modern treatment modalities, or combinations of them, have enormous potential improve quality of life for humans and even other animals afflicted by otitis media in all its forms, and they demonstrate the importance of continuing these kinds of studies in a controlled manner, optimizing or even replacing traditional treatment modalities (e.g., antibiotics).

Future of Animal Studies

The use of animals in teaching, experimentation, and research has decreased over time, although it is still necessary in many areas and situations, as demonstrated above. This need should be carefully considered because animals have been exploited and abused since prehistoric times as sources of food, for sports (e.g., hunting and fights), for labor, sometimes as pets, and so on.

Animal studies provide known/controlled conditions, different groupings for comparisons, and possibilities for longitudinal analysis; however, a large database of previous

animal experiments exists, and computer science simulations based on mathematical models can help reduce this need for using animal. Modern laboratory equipment, developments in biology, the genetic sequencing of microorganisms, in vitro studies on cell cultures that can be evaluated even at the molecular level, and bioluminescence for in vivo analyses of infections [53] can be leveraged or conducted. These in turn will lead to more-focused research and will eventually reduce the need for animal sacrifice.

Prioritizing **reduction** and **replacement** when possible and prioritizing **refinement** and **respect** at all times should pave the way for an ethical future in animal studies. A fourth R, for the word **respect**, should be added to the 3R principle of reduction, replacement and refinement (Arouca law: law no. 11.749 in Brazil [8, 9]), such that it would be renamed as the **4R principle** that guide experimental animal studies.

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