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Prenatal and postnatal developmental studies on the inner ear of the rabbit

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ABSTRACT

Since the inner ear is the main part of the auditory apparatus that is responsible for hearing and balance function, and it is also the first part of the ear to be developed. The current study aimed to investigate its development in the rabbit, as this type of research in this species is very scarce. About 20 fetuses aged 12 to 25 embryonic days (E) and 30 neonatal rabbits from birth (day zero) to 20 days after birth (DAB) were used in this study. Samples were collected and fixed in 10-15% neutral buffered formalin for 48-72 hours and routinely prepared for histological and immunohistochemical investigation. The findings revealed that at E12, the otic placode, which is the embryonic origin of the inner ear, was formed. The placode was invaginated, forming the otic vesicle at E14, which grew and reshaped gradually as the development progressed, as appeared at E17, E20, and E25. Postnatally, the organ of corti, the main structure, continued to develop until reaching its mature form on day 15. The immunohistochemical investigations showed that S100 was a more specific marker for labeling the neurons of the spiral ganglia and the nerve fibers of both cochlear and vestibular nerves than calretinin. In conclusion, the present study revealed that the inner ear development started with the otic vesicle formation on the 14th day of gestation, then grew gradually as the development progressed, and continued till the organ of the corti reached its maturity on the 15th day postnatally, where the rabbit can normally hear.

1. INTRODUCTION

The inner ear is a highly complex sensory organ responsible for hearing and balance function (Donaldson and Duckert, 1991; Torres and Giráldez, 1998). It's the first part of the ear to be developed. Its development is one of the most remarkable events in vertebrate organogenesis, as its three-dimensional structure, in addition to the ganglion that innervates its sensory structures, originates from a simple hollow epithelial sac called the otic vesicle (Torres and Giráldez, 1998; Barald and Kelley, 2004; Mansour and Schoenwolf, 2005).

The inner ear is composed of auditory and vestibular systems, which are formed by two labyrinths, one inside the other, the membranous labyrinth contained within the bony one. The latter consists of the vestibule, the three semicircular canals, and the spirally coiled cochlea. Within each structure a corresponding part of the membranous labyrinth is present: the vestibule has the utricle and saccule, each semicircular canal contains a semicircular duct, and the cochlea has the cochlear duct (Li et al., 2015; (Ekdale, 2016). These membranous structures have the main specific receptors, which are the hair cells. These cells have a vital role in sound transmission as well as motion signals from the periphery to the central nervous system. Neuronal functions such as excitability, response dynamics, and transmission mechanisms are mainly dependent on the concentration of the calcium ions that are controlled by the aid of calcium-binding proteins such as calretinin and S100 (Baimbridge et al., 1992; Schwaller et al., 2002; Fairless et al., 2019).

Calretinin immunostaining is distinctly related to the developing sensory cells, ganglion neurons of the cochlea, and vestibular nerve fibers (Dechesne et al., 1994).

The immunostaining of S100 protein is mainly related to the development of non-sensory cells such as pillars, Deiters' cells of the cochlea, hair cells of the vestibule, and their connecting nerve fibers. Also, it is present in neural crest-derived cells such as Schwann cells, chondrocytes, melanocytes, and intermediate cells of the stria vascularis (Buckiová and Syka, 2009; Johnson Chacko et al., 2016).

The morphological changes during the different developmental stages of the inner ear in the rabbit species are scarcely reported (Retzius, 1884), so the aim of the current work was to provide a detailed morphological source for the rabbit inner ear development histologically and immunohistochemically.

2. MATERIAL AND METHODS

2.1 Animals and tissue sampling:

Twenty rabbit fetuses of embryonic days (E) E12, E14, E17, E20, and E25 (four animals per each age), and thirty neonatal young rabbits at day 0 (day of birth), 4, 7, 10, 15, and 20 days after birth (DAB) (five animals per each age), were used in this study.

Pregnant rabbits weighing 3–4 kg and aged 4–6 months were slaughtered in compliance with recommendations of the animal care committee of the Faculty of Veterinary Medicine, Benha University, Qalyubia Governorate, Egypt (approval number: BUFVTM 10–08-22), then their abdominal cavities were opened, uteri were exposed, then

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incised, and embryos were collected immediately. Fetuses of E12,14, and 17 were wholly preserved in 10-15% neutral buffered formalin for 48-72 hours, while only the heads of E20 and E25 fetuses and those of all neonatal slaughtered rabbits were separated and divided into two halves. The petrous part of the temporal bones was dissected, and the tympanic bullae were opened to remove the ossicles, then the round and oval windows were opened for good perfusion of the neutral buffered formalin.

2.2 Histological preparation:

Following fixation, the bone tissue was softened with the decalcifying agent EDTA, with weekly renewal of the agent until the bone became soft (Malkemper et al., 2020). The tissues were washed under running tap water and exposed to routine histological procedures. Following dehydration with ascending grades of alcohol and clearing with xylene, the prepared sample was embedded in paraffin in a horizontal position. Next, serial sections of 5 micrometers (µm) were obtained from the paraffinized blocks and stained with the Hematoxylin and Eosin stain according to Bancroft and Gamble (2002).

2.3 Immunohistochemical preparation:

Paraffin sections of 5 µm were collected from blocks of postnatal (P) samples (P0, p7, and P15) on positively charged microscope slides, then deparaffinized in xylene, sequentially rehydrated in descending grades of ethanol, and rinsed in phosphate buffered saline (PBS). Antigen retrieval was done by heating the tissue sections in 10 mM sodium citrate buffer (pH 6.0) at 80 °C for 40 minutes, followed by cooling at room temperature for 20 minutes. To reduce endogenous peroxidase activity, sections were incubated with 0.5-3.0 % hydrogen peroxide (H_2O_2) for 10-15 min. Sections were washed with phosphate buffered saline (PBS) and incubated with 10% normal goat serum for 1 hour at room temperature to block non-specific stains. Then sections were incubated with primary antibodies at 4°C in a humidified chamber. The antibodies used in the immunohistochemistry study was illustrated in table 1

Table 1. The antibodies used in the immunohistochemistry study.

Primary antibody	Type	Dilution	Company	Catalog number
Anti- CAR	Anti-human mouse monoclonal antibody	Ready- to-use	Dako	M7245
Anti-S100	Anti-Human rabbit polyclonal antibody	1:1000	Dako	Z0311

3. RESULTS

3.1 The prenatal period

At E12, the primordial otic placode (OP) appeared as a thickening in the surface ectoderm on the eighter side of the developing rhombencephalon (RH) (Fig. 1A).

At E14, a hollow vesicle lined by a stratified columnar epithelium (SCE) known as the otic vesicle (OV) appeared, resulting from the invagination and closure of the placode, from which neuroblast cells were delaminated and coalesced, initially forming the cochleovestibular ganglia (CVG) (Fig. 1B), which divided, as development advanced, into the cochlear spiral ganglia and the vestibular one for innervating the inner ear structures. Then the vesicle underwent a period of growth and re-shaping to form the membranous labyrinth of the inner ear.

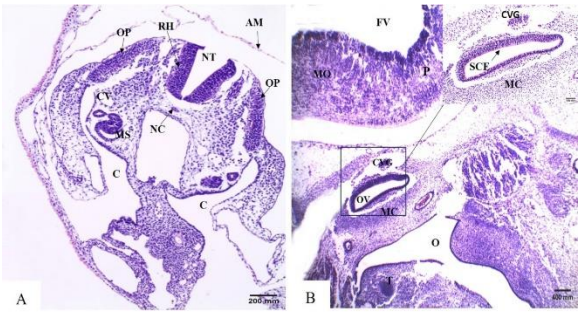

Figure 1 Photomicrographs of a rabbit embryo (A) (C.S) at E12 and (B) (L.S) at E14. NT; neural tube, RH; rhombencephalon, OP; otic placode, NC; notochord, CV, cardinal vein, MS; mesonephros, C; celomic cavity, AM; amnion, OV; otic vesicle, CVG; cochleovestibular ganglia, SCE; stratified columnar epithelium, MC; mesenchyme, FV; fourth ventricle, MO; medulla oblingata, P; pons, O; oropharynx, T; tongue.

At E17, the developing cochlear ducts (CD) with spiral ganglions (SG) appeared.

Note the medial position of the future organ of corti and its innervating ganglia. There were many blood capillaries within the surrounding mesenchymal tissue. At this stage, a cartilaginous (premature bony) otic capsule (OTC) of the cochlea was also observed (Fig. 2A).

At E20, one and half turns with elongated cochlear ducts were observed. In addition, the vestibular structures, including the developing saccule (S) and utricle (U), were also seen. The utriculus had an oval form, while the sacculus appeared more roundish. The lumen of each cochlear tunnel, saccule, and utricle was lined with stratified columnar epithelia (SCE) in the area of the future organ of the corti and sensory macula, respectively (Fig. 2B-D).

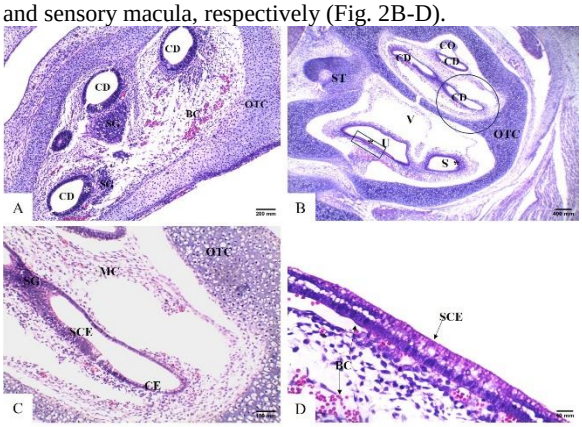

Figure 2 Photomicrographs of the developing inner ear of the rabbit at E17 (A) and E20 (B) with high magnification of the developing cochlear canal (C) and utricular macula (D). CD; cochlear duct, SG; spiral ganglia, OTC; otic capsule, (white asterisk) future organ of corti, CO; cochlea, V; vestibule, U; utricle, S; saccule, (black asterisk) macula, SCE; stratified columnar epithelium, CE; cuboidal epithelium, ST; stapes, BC; blood capillaris, MC; mesenchyme.

At E25, the developing cochlear ducts became triangular in shape. The developing reissner's (RM) and basilar membranes (BM) were formed by the roof and floor of the cochlear duct, respectively, dividing the cochlear canal into three scales, namely from dorsal to ventral: scala vestibuli (SV), media (SM), and tympani (ST). In addition, within the scala media (cochlear duct), the developing stria vascularis (STV), spiral ligament (SL), limbus (L), and immature tectorial membrane (TM) were formed. Centrally, the mesenchymal tissue was invested with blood capillaries, fibers of the developing cochlear nerve CNF), and the spiral ganglia laterally, all these structures forming the future modiolus (MO) (Fig. 3A).

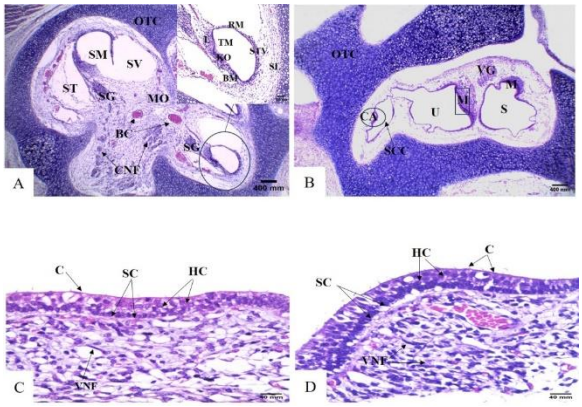

Figure 3 Photomicrographs of the developing cochlea (A) and vestibule (B) of the rabbit inner ear at E25, with magnified photographs of the macula (C) and crista ampullaris (D).SG; spiral ganglia, OTC; otic capsule, SV; scala vestibuli, SM; scala media, ST; scala tympani, STV; stia vascularis, RM; ressieners membrane, TM; tectorial membrane, BM; basilar membrane, KO; kolliker's organ, SL; spiral ligament, L; limbus, CNF; cochlear nerve fibers, MO; modiolus, BC; blood capillaris, U; utricle, S; saccule, SCC; semicircular canal, CA; crista ampullaris (circle), M; macula (rectangle), HC; hair cells, SC; supporting cells, C; cilia, VNF; vestibular nerve fibers.

The spot of the future macula (M) utriculi had a more horizontal position in relation to that of the sacculi, which was vertically located. Also, the semicircular duct appeared with its lumen opened into the utricle (Fig. 3B). The sensory epithelia of Macula and Crista ampullaris (CA) were completely differentiated into mature hair cells (HC) with their cilia (C) apically and supporting cells (SC) basally, and the vestibular nerve fibers (VNF) were also observed (Fig. 3C &D).

3.2 The postnatal period

The main structure of the developing inner ear that continues its development after birth is the organ of Corti.

At day 0 (day of birth) the cochlear duct was lined with immature epithelium (Koelliker's organ (KO). The hair cells, pillar cells, and dieters' cells (DC) were differentiated. The Nuel's spaces (NS) weren't formed yet, so the cells formed a compact layer where they could hardly be distinguished. At this age, the tectorial membrane was firmly adhered to the epithelial surface, the area of the inner spiral sulcus (ISS) was occupied by a tall columnar epithelium, and Vas spirale (SV) was conspicuous (Fig. 4A).

At P4, the organ of Corti continued to develop. At this age, the main morphological changes that were observed such as gradual regression of the epithelial cells in the area of the developing inner spiral sulcus, and the appearance of the first Nuel's space between the outer pillar cell (OP) and the adjacent outer hair cell (Fig. 4B).

At P7, the organ of Corti was approaching its mature form. The cytoplasm was clearer, and the pillar, supporting, and hair cells were slightly distinguished. The inner hair cell (IH) was separated from the outer one by the pillar cells, so the tunnel of Corti obviously appeared (Fig. 4C).

At P10, the Nuel's spaces were clearly visible, so the outer hair cells (OH) within each row are apart. The stratified epithelium in the area of the future inner spiral sulcus largely disappeared, except for a few cells that remained close to the inner hair cell (Fig. 4D).

At P15 and P20, the organ of the corti was in its mature form, where all sensory hair cells, supporting cells, and the corti tunnel were observed. Apparently, the reticular membrane was sloped inward, resulting from elongation of the outer supporting cells. Nuel's spaces were clearly observed (Fig. 4 E & F).

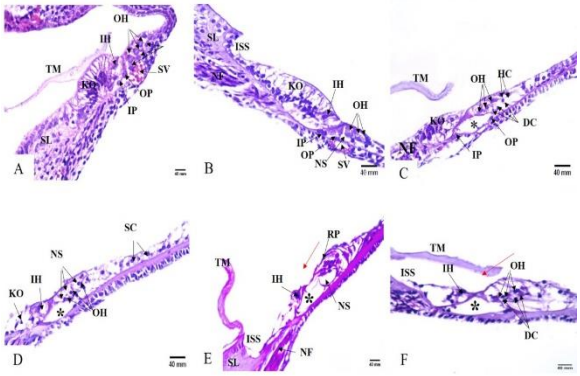

Figure 4 Photomicrographs showing the morphological changes within the organ of corti of the rabbit at P0 (A), P4 (B), P7(C), P10 (D), P15 (E), and P20 (F). KO; kolliker's organ, IH; inner hair cell, OH; outer hair cells, DC; dieter's cells, IP; inner pillar cell, OP; outer pillar cell, SV; spiral vessel, SL; spiral limbus, NS; Nuel's space, TM; tectorial membrane, ISS; inner spiral sulcus, NF; nerve fibers, SC; supporting cells, (*) tunnel of corti, (the red arrow shows inward deviation of RP; reticular plate).

3.3 Immunohistochemistry

3.3.1. Calretinin expression during postnatal development.

At day 0, CaR-cytoplasmic immunoreactivity was moderately present in the inner and outer hair cells, interdental cells of the spiral limbus, and chondrocytes of the osseus spiral lamina (Fig. 5A). The cells of the spiral ganglion and the connecting fibers of the cochlear nerve were weakly stained (Fig. 5B). A strong CaR cytoplasmatic signal was present in the cells of the vestibular macula, in addition to the vestibular nerve fibers (Fig. 5C).

At P7, the outer hair cells were positively labelled, while the inner one wasn't stained (Fig. 5D). The spiral ganglion cells were negatively stained, as were the cochlear nerve fibers (Fig. 5E). A weak reaction was observed in the hair cells of the macula, but the vestibular nerve fibers were strongly stained (Fig. 5F).

At P15, a CaR-reaction appeared in the cochlea, mainly in the inner hair cells and pillar cells (Fig. 5G). The neurons of the spiral ganglion were negatively stained, as were the connecting nerve fibers (Fig. 5H). The hair cells of the vestibular macula were negatively stained, but moderate staining was observed in the nerve fibers compared with P0 and P7 (Fig. 5I).

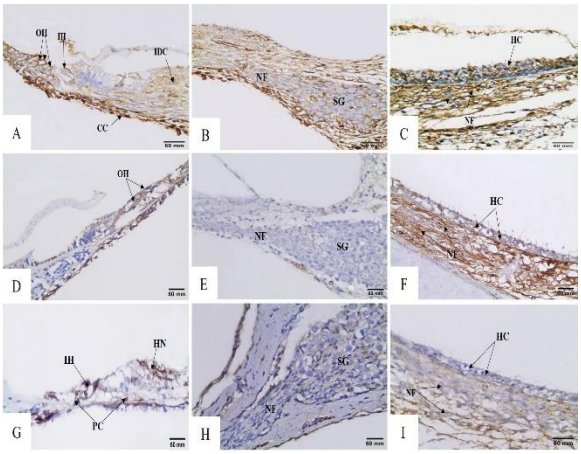

Figure 5 Photomicrographs of calretinin expression during postnatal development of the rabbit inner ear at P0 (A, B and C), P7 (D, E and F) and P15 (G, H and I) show the organ of corti (A, D and G), cochlear nerve fibers and spiral ganglion neurons (B, E and H), and vestibular macula (C, F and I). IH; inner hair cell, OH; outer hair cells, IDC; interdental cells, CC; chondrocytes of the osseus spiral lamina, HN; Hansen's cells, PC; pillar cells, SG; spiral ganglia, HC; hair cells, NF; nerve fibers of cochlear and vestibular nerves.

3.3.2. S100 expression during postnatal development

At day 0, A strong S100 cytoplasmic staining was present in cells of Koelliker's organ, hair cells, and pillar cells (Fig. 6A). Neurons in the spiral ganglion expressed strong staining for S100 in the cytoplasm and also in the cochlear nerve fibers (Fig. 6B). A strong stain was present in the vestibular sensory hair cells and their connecting nerve fibers, supporting cells, the otolithic membrane (OM), and otoconia (Fig. 6C).

At P7, the interdental cells (IDC) of the spiral limbus, dieters, and pillar cells were positively immunoreactive (Fig. 6D). Within the spiral ganglion, the cytoplasm of cochlear nerve neurons was strongly labelled in addition to the connecting nerve fibers (Fig. 6E). In the vestibular apparatus, both cytoplasmic and nuclear S100 immunolabelling was markedly seen within the hair and supporting cells of the sensory macula, in addition to the vestibular nerve fibers and otolithic membrane (Fig. 6F).

At P15, S100 was expressed mainly in the inner hair cells, pillar cells, interdental cells, and dieter's cells of the cochlea (Fig. 6G). The nerve bundles and ganglion neurons were positively stained (Fig. 6H). A strong immunolabelling was present in the hair cells, supporting cells of the vestibular macula, and their nerve fibers (Fig. 6 I).

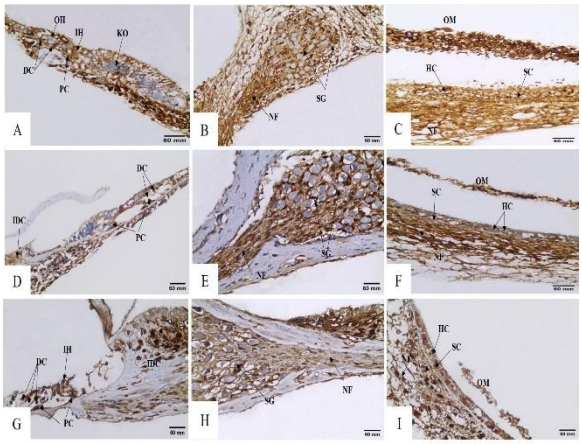

Figure 6 Photomicrographs of S100expression during postnatal development of the rabbit inner earat P0 (A, B and C), P7 (D, E and F) and P15 (G, H and I) show the organ of corti (A, D and G), cochlear nerve fibers and spiral ganglion neurons (B, E and H), and vestibular macula (C, F and I). KO; kollikers organ, DC; dieter's cells, IH; inner hair cell, IDC; interdental cells, PC; pillar cells, SG; spiral ganglia, OM; otolithic membrane and otoconia, HC; hair cells, SC; supporting cells, NF; nerve fibers of cochlear and vestibular nerves.

4. DISCUSSION

The development of the organs necessary for hearing and balance function is a complex and interwoven process. Awareness of the inner ear developmental process and factors involved in it alerts the surgeon to anatomical associations and explains important deviations from normal. So, our study focused on rabbit inner ear development.

The rabbit inner ear was developed from the otic placode at E12, while this placode was formed at E8 (8.5–8.75) day post coitus (DPC) in mouse, 9.5 DPC in rat, and around the 3rd week of gestation in humans. This placode underwent invagination then closure forming a hollow epithelial spherical sac adjacent to the neural tube called the otic vesicle that appeared at E14, while this vesicle was formed between 8.5–9.25 day post coitus (DPC) in mouse, 9.5–10.25 DPC in rat, and at around 4th week of gestation in humans (Torres and Giráldez, 1998; Whitfield, 2015). The differences in the time of the otic placode formation between species are mainly coinciding with the appearance of other embryonic structures, particularly the neural tube and the hindbrain, as they provide factors responsible for the placode induction and also the subsequent patterning as reported by Fritzsch and Beisel (2001), Fekete and Wu (2002), and Ohyama et al. (2007). By E17, the rabbit cochlea was developed and consisted of ducts lined with pseudostratified columnar epithelia in the area of the future organ of corti, similar results have been reported in rats by Balcioglu et al. (2021). At E20, the utriculo-saccular ducts appeared, while in rats, their development started at the second week of gestation (Powles-Glover & Maconochie, 2018).

At E25, the rabbit membranous labyrinth attained its mature shape, where its main structures were formed, whereas the mouse labyrinth was matured at E17 (Morsli et al., 1998).

The main structure of the inner ear that continued maturation after birth was the organ of the corti. The main morphological changes that occurred were the tectorial membrane detachment, the tunnel of corti formation, and the opening of the inner spiral sulcus. These changes occurred in the rabbit between 4 and 15 DAB, which is similar to those reported in rats, between 8 and 12 DAB (Roth and Bruns, 1992; Balcioglu et al., 2021) and mouse, between 5 and 10 DAB (Kraus & Aulbach-Kraus, 1981).

Our study demonstrated the expression of calretinin and S100 proteins in the cochlear and vestibular structures during the postnatal development of the rabbit inner ear.

Concerning the developing cochlea of the rabbit, the CaR-immunostaining was exclusively present in sensory cells and also was found in non-sensory cells such as Deiters', Hensen's and interdental cells. On the other hand, the spiral neurons and the cochlear nerve fibers weren't showing any staining. The CaR- expression in the developing sensory cells of the rabbit cochlea were similar to those reported by Dechesne et al. (1994) in mouse. S100 immunoreactivity was specifically expressed in the nerve fibers and spiral ganglion cells of the cochlear nerve and markedly expressed in non-sensory cells such as pillar, Deiters', and interdental cells of the spiral limbus, this was similar to that reported by Buckiová and Syka (2009) in mice.

Regarding vestibular macula development, CaR- was weakly expressed in supporting cells while showed moderate cytoplasmic staining in hair cells and the underlying vestibular nerve fibers. On the other hand, S100 showed strong cytoplasmic and nuclear reactions in all structures of the macula, including sensory, supporting cells, otoconial membrane crystals, and the afferent vestibular nerve fibers. Active calcium transport is essential for the production of calcium carbonate (otolith), which are biomineralized ear stones that contribute to both hearing and vestibular function in fish, so S100 might be involved in this process. The expression of CaR- and S100 within the vestibular nerve fibers might be related to the myelination of the nerve fibers. All these results were nearly similar to those reported by Buckiová and Syka (2009) in mice. This study revealed that the sites of CaR- and S100 expression in the rabbit inner ear during early postnatal development showed independent patterns with only limited overlap which may be useful in detecting various degenerated tissues in the inner ear.

5. CONCLUSIONS

Like other mammals, our study revealed that the otic vesicle was the embryonic origin for the rabbit inner ear. Nearly all inner ear auditory and vestibular structures were completely developed before birth except the organ of the corti, which continued to develop within two weeks after birth. So, our study provided a good source for understanding the

development of the rabbit inner ear, including mainly the cochlea and the vestibule. Further studies are needed to investigate the development of semicircular canals.

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