IMAGE ANALYSIS OF NEURAL AND IMMUNE MARKERS DURING THE INNER EAR DEVELOPMENT OF RAT FETUSES WITH CONGENIT DIAPHRAGMATIC HERNIA

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INTRODUCTION.

Sensorineural hearing loss (SNHL) is a clinical heterogeneous disorder that appears in a high percentage of the patients with congenital diaphragmatic hernia (CDH) (between 44% and 100% depending on the study).(1) Alterations in the immune response (IR) to infections or trauma seem to be related to SNHL, therefore, we have considered whether hearing loss in patients with CDH is also related to IR, since it seems to be due not only to the treatments received but to another malformation associated with the CDH (1) (2). The majority of hearing disorders occur due to the death of either inner ear hair cells (HCs) or spiral ganglion neurons (SGNs), thus leading to SNHL. Our main objective is to find a relationship between immune system altered responses, neuronal spiral ganglion ontogeny and CDH in developing inner ear using our CDH rat model induced with nitrofen. In the present study, we analyze with immunohistochemistry techniques and Image J software the levels of neural and IR markers to establish a relationship with CDH pathology.

MATERIAL AND METHODS.

Pregnant rats were treated on E9.5 with either nitrofen (1mg/250 g i.p., CDH) or olive oil alone (Control group) and the fetuses were recovered on E15, E18 and E21. Embryonic parafined inner ear was cut (5 μm) and stained following a standard immunohistochemistry protocol with a mouse antibody against CD68 (dil. 1/300) and rabbits policlonal antibodies anti NeuN (1/1000) and anti CD20 (1/. Sections were stained also with Haematoxilyne. Images of the inner ear were taken with Image ProPlus 6.3 software and analyzed with Fiji Image J software (3). ROI areas were selected from control and CDH group in NeuN immunohsitochemistry. Statistical analysis was made with t-student method. using GraphPad Prism 7 software.

RESULTS

Figure 1

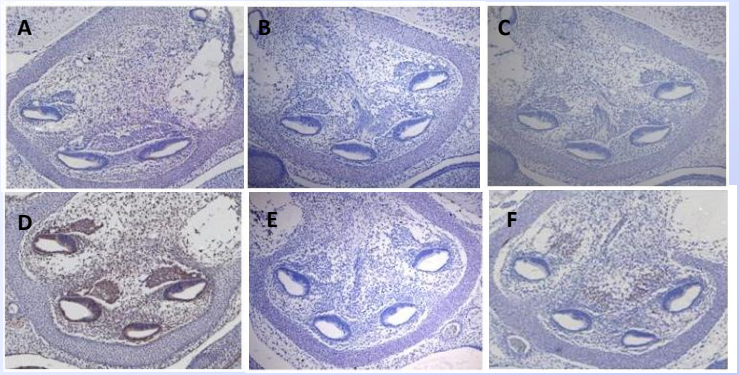

Fig.1. Expression of neural and immune markers observed in cochlear sections by immunohistochemistry technique,

Representative images of E18 cochleas from control (A,B,C) and nitrofen treated (D,E,F) groups NeuN (C,F). CD206 (A,D) and CD68 (B,E) antibodies are antigen markers of neuronal, M1 and M2 macrophagues, respectively. The expression increases during cochlear development but only C206 has the higher expression in E18 CDH group (D). CD206 expression was mainly located at spiral ganglio, less signal was found in Corti's organ and modiolus (D). CD68 higher expression was mainly located at cochlear modiolus .(E) whilst NeuN expressiion is also loicated at spiral ganglios (F) both in CDH groups. Images were obtained with a BX41 Olympus microscope and ImageProPlus 6.3 acquisition and analysisi software. Objective magnification, 100x.

Figure 2

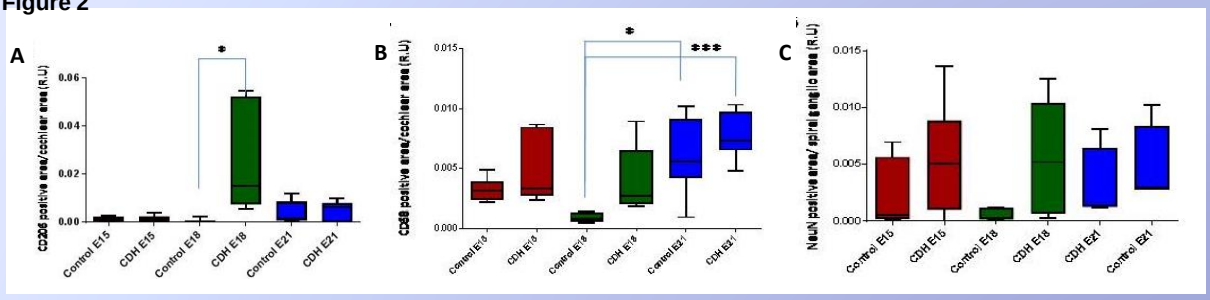

Fig.2. Analysis of expression of neural and immune antigen markers

The area of brown cells were measured in cochlea (n=3) using Fiji software with deconvolution and threshold plugins. The antibody positive index was calculated using the mean value of area obtained by Image J software. **The i**ndex was calculated for each group with the positive area (stained cells) related to the normalized area of the cochlea (cochlear area/inner ear area) in order to correct the size differences between control (healthy) and CDH groups. CD68 (B) and NeuN (C). All healthy groups shows a tendency to increase during cochlear development . Increased level of CD68 is observed along inner ear development with a higher expression at E21 fetus in both groups. The higher expression is found at CDH E18 groups with CD206 (A) and NeuN (C) antibodies. Statistical analysis was made with t-student method.

CONCLUSION

Fiji software has showed to be a potent tool for immunohistochemistry analyse, furthermore analyses are needed to adjust and accurate automatized measure with Fiji. There are changes in the immune response in the ear of fetuses with CDH, there is an increase in the activity of M2 and M1 and the macrophage activity changes towards M2, but the activity of neutrophils does not change. On the other hand, an increase in the number of new neurons is observed in the cochlear ganglia of fetuses with CDH: These data could point, together with the high M2 response, that sensorineural deafness in CDH is caused by defects that occur during embryonic development.

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