

**REGULATION OF THE IMMUNE RESPONSE
IN THE CHILDREN AGED 7-11 YEARS
WITH CHRONIC SENSORINEURAL HEARING LOSS**

**REGULACJA REAKCJI IMMUNOLOGICZNEJ
U DZIECI W WIEKU 7-11 LAT Z PRZEWLEKŁYM
NEUROSENSORYCZNYM UBYTKIEM SŁUCHU**

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STRESZCZENIE

Obustronny neurosensoryczny ubytek słuchu jest czynnikiem wpływającym na wszystkie układy organizmu. Między innymi wpływa na układy endokrynologiczny i immunologiczny. Podwyższony poziom adrenaliny i 17-ketosteroidów świadczy o obecności reakcji stresowej u dzieci w wieku 7-11 lat z przewlekłym obustronnym neurosensorycznym ubytkiem słuchu. Podwyższony poziom tych hormonów powoduje zmiany w układzie immunologicznym. Adrenalina i 17-ketosteroidy aktywują leukocyty, powodując przyspieszenie ich metabolizmu i produkcję przeciwzapalnych interleukinów. Te ostatnie wpływają na limfocyty B, które aktywują się i przekształcają w komórki plazmatyczne. Interleukiny (IL-4) zwiększają liczbę limfocytów Th (helperów) typu Th2. Interleukiny wpływają na limfocyty B, które wytwarzają immunoglobuliny. We krwi dzieci z przewlekłym obustronnym sensorycznym ubytkiem słuchu wykazano podwyższony poziom immunoglobulin A i G oraz obniżony poziom immunoglobuliny M. Natomiast w ślinie badanych dzieci stwierdzono wysoki poziom przeciwciał IgG oraz niski IgA i IgM. Brak równowagi pomiędzy poziomem immunoglobulin a zmianami metabolicznymi leukocytów powoduje osłabienie nieswoistej odporności.

Słowa kluczowe: obustronny neurosensoryczny ubytek słuchu, granulocyty, fosfataza zasadowa, białka kationowe, mieloperoksydaza, adrenalina, 17-kortykosteroidy, interleukiny

Key words: sensorineural hearing loss, granulocytes, alkaline phosphatase, cationic protein, myeloperoxidase, adrenaline, 17-korticosteroids, interleukins

INTRODUCTION

Hearing loss is an impairment which can cause some serious predicaments. There are different types of hearing loss with distinct characteristics. Hearing loss is usually categorized according to the affected area of the hearing pathway. Identifying the type of hearing loss is crucial for effectively treating hearing loss. The types of hearing loss include: a) sensorineural hearing loss (SNHL); b) mixed hearing loss; c) functional hearing loss; d) conductive hearing loss; e) central hearing loss. Genetic hearing loss may be present at birth (congenital) or may progress in either childhood or adulthood. About 50% of congenital hearing loss is genetic and about 50% is acquired. Genetic hearing loss may appear as an isolated finding or as part of a syndrome. About 70% of genetic hearing loss is nonsyndromic and about 30% is syndromic. Different symptoms occur in different stages of a person's life and there are those which are common and there are those which are rare. It is suggested that 4.2% of the world's population has serious hearing defects. Experts of the World Health Organization predicts an increase in this number by 30% (Zolotova, Panchenko 2010).

SNHL is a common disorder that affects millions of people. Hearing loss has many different presentations, ranging in severity from mild to profound, including low- and high-pitch patterns, and can affect people of any age. SNHL is caused by dysfunctions in the inner ear or the nerves surrounding the inner ear. Brain malfunctions can also lead to sensorineural hearing loss. Bilateral hearing loss is the type of hearing loss where the hearing impairment affects both the ears. SNHL not only involves a reduction in sound level, or ability to hear faint sounds, but also affects speech understanding or ability to hear clearly. It cannot be corrected medically or surgically. It is a permanent loss. It's threat to the child's mental development also. School-aged children have substantial adverse effects on school performance and social interactions (Schmidt 1984).

The role of the immune system in mediating inner ear pathology has received considerable attention over the past two decades. Gylling (1969) first proposed the connection between the immune system and SNHL. Ten years later was proposed the term "autoimmune disease of the inner ear" (McCabe 1979). Today use the term "immune-mediated reduction of hearing" (Broughton et al. 2004). They comprise certain forms of fluctuating or rapidly progressive SNHL with or without endolymphatic hydrops. Immune-mediated audio-vestibular dysfunctioning is either a separate disease entity or part of a more generalized (auto-) immune process. The various attempts which have been made to develop methods or tests to confirm the diagnosis of immune-mediated SNHL are critically reviewed, including the treatment responses to immunosuppressive therapy (Veldman 1998).

Many papers summarized recent basic science research into the pathogenesis of autoimmune inner ear disease, review the current diagnostic work-up for patients with suspected ear disease, and delineate treatment strategies. For example, autoimmune sensorineural hearing loss (ASNHL) typically produces a bilateral rapidly progressive loss of hearing that may occur suddenly. Although autoantibodies and autoreactive T cells have been implicated in the etiopathogenesis of ASNHL, several central issues remain unresolved, including the relative prominence of B cell or T cell

autoimmunity in the initiation and progression of ASNHL, the identity of the putative inner ear self-antigen(s) that target ASNHL, and the development and application of immunosuppressive therapies for preventing the progressive hearing loss which may be profound and require cochlear implantation (Solares et al. 2003).

Patients with congenital hearing loss possess sensitized T cells, antibodies to myelin and neuro-specific enolase (Elies 1983, Arnold et al. 1985, Moskalenko et al. 1989, Timen et al. 2002, 2004, Melnikov et al. 2003, Zolotova 2004, Chashcheva 2003). There are circulating antibodies to nerve growth factor in the blood. Its levels are higher than in persons who lost hearing before (Zolotova, Panchenko 2010). This is due to inadequate permeability of the hematoencephalic barrier (Reddy et al. 2005, Tan, Shepherd 2006). In the long-term SNHL, the blood level of T-helper cells was higher (Chashcheva 2003). There is suggesting that the progression of sensorineural hearing loss is associated with activation and degranulation of neutrophilic granulocytes in local ischemia areas due to violations of the blood-brain barrier (Malinvaud et al. 2006). It has been considered that microglia is a mediator in these processes allocating a large amount of cytokines to blood (Hrebenyuk 2007). Of the myriad proteolytic enzymes implicated in the development of disease, neutrophil elastase has undoubtedly some of the most versatile effects. Although its key physiologic role is in innate host defense, it can also participate in tissue remodeling and possesses secretagogue actions that are now recognized as important to local inflammatory responses (Chua, Laurent 2006). Human neutrophils contain high levels of the serine protease elastase, which is stored in azurophilic granules and is secreted in response to inflammatory stimuli. Elastase is capable of degrading many components of extracellular matrix and has cytotoxic effects on endothelial cells (Bless et al. 1997). An increased level of leukocyte elastase provokes the inner ear cells to apoptosis in patients with SNHL (Bless et al. 1997, Chua, Laurent 2006).

Immune reactivity plays a more important role in the etiopathogenesis and natural course of various inner ear disorders than was thought originally. Therefore, we hypothesized that children with SNHL have different activity of immune system, as well as hormonal profile compared to healthy children. The aim of the present study was to assess the role of immune system and hormones level in the children with SNHL. We determined an adrenaline and 17-ketosteroids levels in the daily urine samples, phagocytic index, phagocyte number, typical reaction of granulocytes to the alkaline phosphatase, myeloperoxidase, phospholipids' and cationic proteins' determination, as well as immunoglobulin's and interleukin's levels in the blood and saliva of the healthy children and children affected by SNHL.

MATERIALS AND METHODS

Study groups. Healthy and SNHL affected children, 7-11 years old, were acquired from the special secondary school № 29 for children with diminished hearing in Kherson (Ukraine). Comparison of data was performed with children from schools № 13 and № 50 in Kherson. All procedures were carried out in accordance with the guidelines and were authorized by the Commission on Ethics in Scientific Research by the Kherson State University. The study group consisted of 60 children

with SNHL (27 girls, 33 boys). Diagnosis of SNHL (III-IV degree) complicated by bilateral cochlear neuritis was made on the basis of clinical history. Sixty healthy subjects were examined as a control group.

Samples. Blood was obtained in tubes with sodium citrate. Do not use samples that yielded hemolysis. After rinsing the mouth saliva was collected in plastic containers and immediately frozen. Urine was collected for 24 hours in a container of 15 g preservative solution (citric acid). In the study, 20 ml. of total urine volume was used.

Hormones assay. The number of daily servings of adrenaline in the urine was determined by calorimetric method using Folin reagent (Kucherenko et al. 1988). In gauge test tubes on 10 ml. added to 1 ml. of urine. In one test tube added standard solution of adrenalin (productions of pharmaceutical company “Zdorovie”, Ukraine) with a concentration 0.04 mg. in 1 ml. After it, in every test tube added for 4 ml. of solution the natrium of carbonate (10%) and 0.5 ml. of Folin reagent. Content of test tube was mixed. In 5 minutes of volume test tubes led to solution the natrium of carbonate to 10 ml. (w = 10%) and mixed again. Photometry was conducted in cuvettes with long by ways a 10 mm, long waves – 670 nm against control (there is standard solution of adrenaline).

Level 17-ketosteroids were also determined by a calorimetric method using M-dinitrobenzene (Shevryakov et al. 2003). Since the beginning of the tubes was introduced into 20 ml. of daily urine samples, 30 ml. (w = 35%) hydrochloric acid, 0.2 ml. of formaldehyde (40%). The tubes are heated for 15 minutes. After hydrolysis, the tube was cooled and the contents were transferred to a separatory watering with 10 ml. of ethyl ether. Extraction was carried out twice for 1.5 minutes. The ether extract of 17-ketosteroids 3 times for 3 minutes was washed with sodium hydroxide (w = 10%). The upper layer, which contained estrogen removed. The extract was washed with 10 ml. of distilled water. The ether extract was evaporated at 50 degrees to dryness. To the dry residue was added 0.2 ml. of ethanol (95.5%), 0.2 ml. of M-dinitrobenzene in ethanol, 0.2 ml. of potassium hydroxide in methanol. The mixture was left for 1 hour in the dark. After the appearance of color, 3 ml. of ethyl alcohol (50%) and 2 ml. of chloroform were added. The upper layer of the alcohol was removed and 1 ml. of ethanol (95.5%). Photometry was performed in cells with a long path of 5 mm, wavelength – 530 nm versus control (0.2 ml. of M-dinitrobenzene, 0.2 ml. of ethanol (95.5%), 0.2 ml. of potassium hydroxide in methanol).

Phagocytic assays involving *S. cerevisiae*. Active phagocytosis was determined using culture *S. cerevisiae* (Frimel 1987, Karaulov 2002). In microtube added 0.1 ml. sodium citrate (4%), 0.2 ml. of blood, 0.1 ml. of a suspension of microorganisms *S. cerevisiae*. After mixing the tubes were placed in an incubator (37°C). After 30 minutes, making smears on glass slides and stained by the method of Romanovsky-Giemsa. Preparations looked under oil immersion lens and counted the number of white blood cells that are involved in phagocytosis (phagocytic index) and the average number of absorbed microbial one phagocyte (phagocyte number).

Granulocyte's metabolic state assessment. Metabolic state of granulocytes was evaluated using the methods of cytochemistry in principle Astaldi (Astaldi, Verga 1957). To account for the results of cytochemical studies used semi-quantitative method of assessing results on the principle Astaldi-Verga, which is based on identifying the varying intensity of specific color. Depending on this, the treated cells

were divided into 4 groups: group-negative (-), weakly positive (+), positive (+ +), strongly positive (+ + +). To quantify the results the 100 white blood cells, differentiating them on that principle. Then the number of white blood cells with the same color intensity was multiplied by the corresponding number of pluses that group, the sum of these products is equivalent units. The sum of the values divided by 100 is the average rate for the cytochemical single cell.

Alkaline phosphatase activity assay. Alkaline phosphatase activity was determined by the method of Keploy (Menshikov et al. 1987). The method is based on the phenomenon of splitting alkaline phosphatase α -naphthyl phosphate release α -naphthol, which reacts with a diazonium salt. As a result, there is a deposit of neutrophil cytoplasm dye purple. Dried blood spots were fixed in 10% neutral formalin and methanol, 30 seconds. After that, the drug is applied incubation medium (10 minutes) (35 mg α -naphthyl phosphate, 35 mg of solid blue dye RR, 35 ml. of propanediol buffer (0.05 M, pH 9.75)), and rinsed in running water for 10 minutes.

Myeloperoxidase activity assay. The myeloperoxidase activity determined using benzidine method of Graham and Knoll (Menshikov et al. 1987). The method is based on the principle of conversion of benzidine brown oksibenzidin. Fresh blood smears were fixed in formalin-alcohol solution (30 seconds). After this, preparations were washed in running water and dried. Medications filled with a mixture of benzidine (4 mg), 6 ml. of ethanol (96%), 4 ml. of distilled water, 0.02 ml. of hydrogen peroxide (3%) for 5 minutes. After washed in running water and stained with paint Romanovsky-Giemsa.

Cationic proteins level assay. The content of cationic proteins was determined by the method of Shubich (Shubich et al.1981). The method is based on the selective staining of cationic proteins bromophenol blue. Blood smears were fixed races are happening sulfosalicylic acid (5%) 90 seconds. Washed with distilled water and dried. After drying, were stained with 0.1% solution of bromophenol blue in borate buffer (pH 8.2) for 1-2 minutes. After this, preparations were washed three times with buffer (pH 8.2) for 3 minutes. For contrast-dried preparations were stained with safranin. Cationic protein in the neutrophils cytoplasm detected in the form of blue beads.

Phospholipids level assay. Phospholipid content was estimated by the method of Goldman (Menshikov et al. 1987). The method is based on the staining of neutral fat by Sudan-III in orange. Blood smears were fixed in formalin and alcohol (1:4). After staining in a Sudan-III solution (ethanol, distilled water- α , naphthol, Sudan-III) preparations were placed in ethanol (70%).

Immunoglobulins and interleukins assay. We used ELISA to determine IgA, IgM, IgG levels in serum and saliva, as well as interleukins IL-2, IL-4, IL-5, IL-10, and IL-13. Immunoglobulin levels were determined using a set of reagents ELISA firm "Granum" (Kharkiv, Ukraine). Blood was collected in a vacuum tube «Vacuete» with activator folding. Indicators of immunoglobulins expressed in grams per liter. Quantify levels of interleukin whey of blood and saliva was performed using reagent kits "ProCon IL-2", "ProCon IL-4", "ProCon IL-5", "ProCon IL-10", "ProCon IL-13" production "Protein Contour" (St. Petersburg, Russia).

Statistical and graphical analysis. All statistical calculation and graphical analysis of the data were performed on separate data from each individual with

STATISTICA 8.0 software. Results are expressed as mean \pm S.E.M. Results are presented as mean and median. Significance of differences in the TBARS level, enzymes activities and oxygen consumption data (significance level at $p < 0.05$) was examined using Mann-Whitney U test. Significance of the means in the group was determined using the Wilcoxon test. In addition, the relationships between data in each group were evaluated using Spearman's correlation analysis (Lapach et al. 2001, Novikov, Novochadov 2005).

RESULTS AND DISCUSSION

The first step of our study was to determine the level of the daily excretion of adrenaline and 17-ketosteroids in urea samples. It is possible to establish the presence of stress reactions in the children with SNHL. Thus, the adrenaline level was higher (46.04 μg per day) in the children with SNHL compared to those of the control group (16.78 μg per day) (Fig. 1). The children with SNHD have higher adrena-

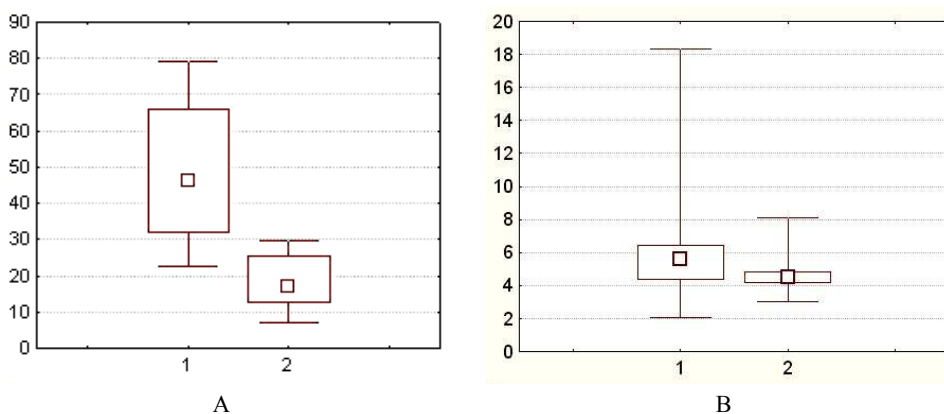


Fig. 1. The median of adrenaline (A) and 17-ketosteroids (B) levels in the daily urine (μg per day) in the children with SNHL (1) and in the control group (2), ($p \leq 0.05$, $n = 60$)

Ryc. 1. Mediana poziomu adrenaliny (A) i 17-kortykosteroidów (B) w moczu dobowym (mkg na dobę) u dzieci z przewlekłym obustronnym ubytkiem słuchu (1) i w grupie kontrolnej dzieci zdrowych (2), ($p \leq 0,05$; $n = 60$)

line and 17-ketosteroids levels. It is suggested that these higher stress-related hormones levels are rather the result than the cause of this chronic disease. The exact impact of these hormones' levels on the inner ear and endolymph homeostasis is yet unknown. Evidence accumulated over the last 20 years suggests that norepinephrine (NE) fulfills the criteria for neurotransmitter/neuromodulator in lymphoid organs. Thus, primary and secondary lymphoid organs receive extensive sympathetic/noradrenergic innervation. Under stimulation, NE is released from the sympathetic nerve terminals in these organs, and the target immune cells express adrenoreceptors. Through stimulation of these receptors, locally released NE, or circulating catecho-

lamines such as epinephrine, affect lymphocyte traffic, circulation, and proliferation, and modulate cytokine production and the functional activity of different lymphoid cells (Elenkov et al. 2000).

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Numerous cells and proteins are involved in the innate immune response including phagocytes (neutrophils and macrophages), dendritic cells, and complement proteins. Phagocytes are primarily responsible for phagocytosis, the process by which cells engulf and eliminate invading pathogens. Complement proteins function to identify and opsonize (coat) foreign antigens, rendering them susceptible to phagocytosis. Phagocytic index is the average number of bacteria ingested per leukocyte of the patient's blood. Activity of phagocytic system in the children with SNHL also differed. Median of phagocytic index in the group of children with SNHL was 70.8%, and was lower than in those from the control group (93.8%) (Fig. 2A). Phagocytic number was also lower in children with SNHL (3.6%) compared with the control group (10.4%) (Fig. 2B).

There is increasing evidence that immunomodulatory cells, especially T lymphocytes, might be involved in SNHL. Kassner et al. (2011) suggest an enhanced extravasation of proadhesive and proinflammatory lymphocytes from the peripheral circulation, which may contribute to SHL disease induction as well as progression. Patients with acute SNHL revealed elevated plasma levels of soluble sCD40 and sCD40L ligand and a significantly decreased percentage (36%) of lymphocytes, es-

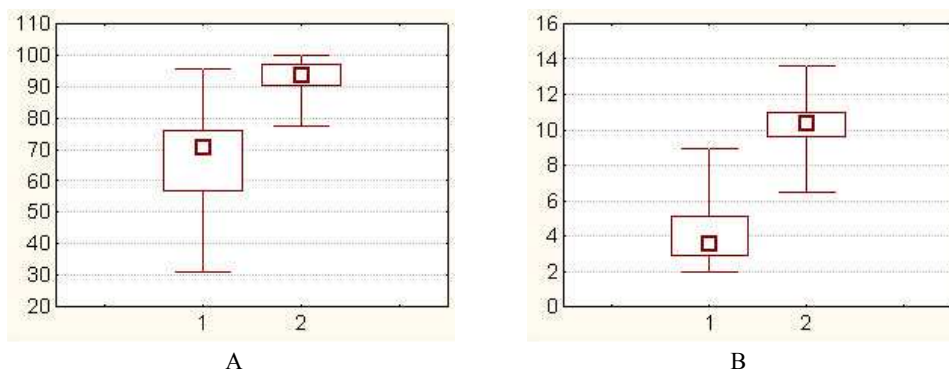


Fig. 2. The median of phagocytic index (A) and phagocyte number (B) in the children with SNHL (1) and the children from the control group (2) ($p \leq 0.05$, $n = 60$)

Ryc. 2. Mediana współczynnika fagocytycznego (A) i liczby fagocytycznej (B) u dzieci z przewlekłym obustronnym ubytkiem słuchu (1) i w grupie dzieci zdrowych (2) ($p \leq 0,05$, $n = 60$)

pecially of T lymphocytes (28%). Additionally, in patients with acute SNHL the percentage of proinflammatory CD40, tumor necrosis factor alpha (TNF- α), cyclooxygenase-2 or CD38-positive T or B lymphocytes was significantly increased (Kassner et al. 2011).

Metabolic activity of granulocytes also differed in the blood of the children with SNHL. Neutrophil function relies largely on the ability of the cell to mobilize its different granules and vesicles to the cell surface and thereby expose and/or release effector molecules to the surrounding tissue. A range of specific markers for the classical granules is available, but finding optimal markers for the secretory vesicles and plasma membrane has historically been more challenging. Latent and non-latent alkaline phosphatase activities are often used to distinguish these two light membrane structures (Pellme et al. 2007). Boxer and Smolen (1988) have given a detailed description of the characteristics of the exocytotic responses of neutrophils to a variety of stimuli. Secretion of the contents of the two types of granules follows different rules. Azurophil granules are discharged only when neutrophils interact with phagocytizable particles, whereas specific granules are discharged on interaction of the cells with both particulate and soluble stimuli. In the latter case, specific granule constituents are released directly into the extracellular environment, demonstrating that these granules can function as secretory organelles (Boxer, Smolen 1988).

Polymorphonuclear neutrophils are implicated in an inflammatory response of the host. The adhesion of neutrophils to the vessel wall and extracellular matrix proteins is required for neutrophil transendothelial migration to the sites of inflammation (Bednarska et al. 2006). Neutrophil alkaline phosphatase (NAP) is used as a marker for granulocyte maturation. NAP activity first appears in myelocytic stages, and increases with granulocytic maturation (Chikkappa 1992). Alkaline phosphatase also takes part in the transmission of signals inside the cell (Pellme et al. 2007). Alkaline phosphatase in the group of children with SNHL was higher compared to

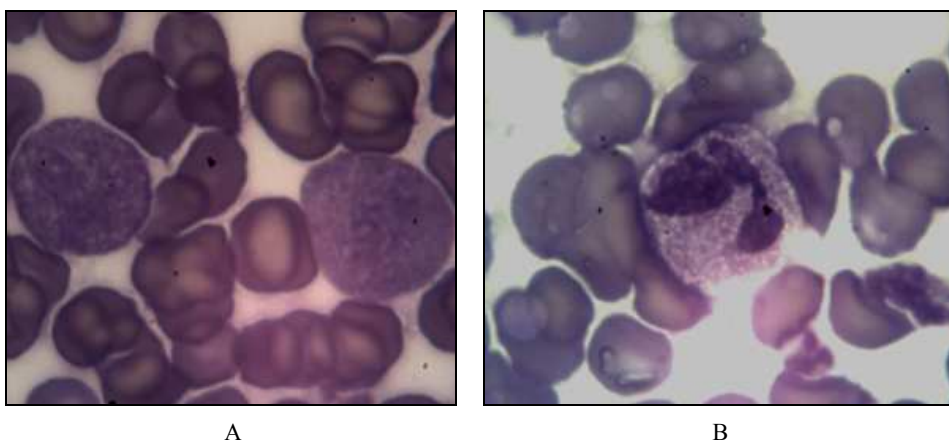


Fig. 3. Typical reaction to the detection of alkaline phosphatase in peripheral blood granulocytes of the children with SNHL (A) and the children of the control group (B)

Ryc. 3. Reakcja typowa leukocytów na fosfatazę zasadową u dzieci z przewlekłym obustronnym ubytkiem słuchu (A) i w grupie dzieci zdrowych (B)

group of healthy children. Amount of the reaction products in granulocytes of children with hearing loss of blood was very high. The nuclei of leukocytes were very poorly painted. Dimensions of leukocytes in children with hearing loss were smaller. Very often, the nuclei of granulocytes have been many segments (Fig. 3).

Eosinophils contain four principal cationic proteins, major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) (Abu-Ghazaleh et al. 1992). ECP and MBP are potent helminthotoxins while EDN is less so. Both ECP and EDN possess neurotoxic and ribonuclease activities. The ECP nucleotide sequence shows similarity to EDN, rat pancreatic ribonuclease, and human angiogenin; all are members of the ribonuclease gene superfamily (Barker et al. 1989). These proteins are mainly expressed in eosinophils, but that certain ones are present in basophils and neutrophils (Abu-Ghazaleh et al. 1992).

ECP is a heterogeneous protein with molecular weights of the variants from 16-24 kDa. ECP has a variety of biological activities interacting with other immune cells and plasma proteins such as coagulation factors and proteins of the complement system. The cytotoxic activity, however, is the most conspicuous. The different isoforms of CP seem to have different biological properties with respect to cytotoxicity and the effects on fibroblasts (Venge, Byström 1998). In our study, the ECP level in leukocytes of the children with SNHL was also lower compared to healthy children (0.9U vs. 1.25U) (Fig. 4).

Phagocytes respond to stimulation with a burst of oxygen consumption, and much, if not all, of the extra oxygen consumed in the respiratory burst is converted first to the superoxide anion and then to hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO), which is released from cytoplasmic granules of neutrophils and monocytes by a degranulation process, reacts with the H_2O_2 formed by the respiratory burst to form a complex that can oxidize a large variety of substances. Among the

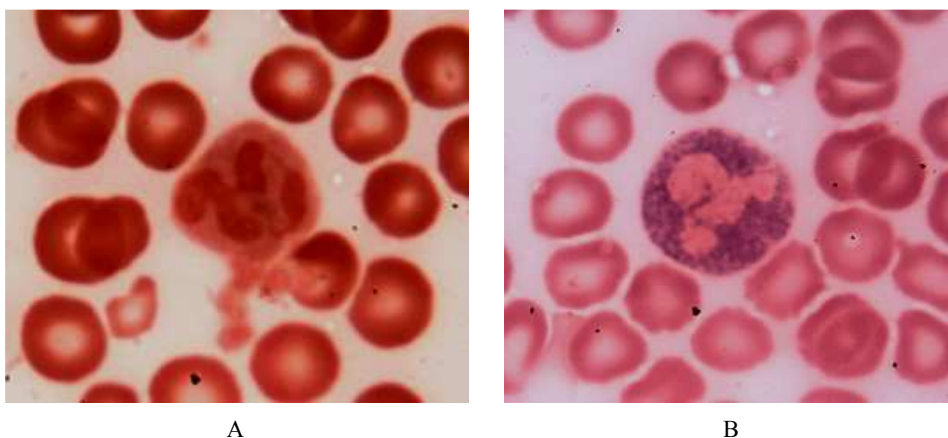


Fig. 4. Typical reaction to the detection of cationic protein peripheral blood granulocytes of the children with SNHL (A) and the children of the control group (B)

Ryc. 4. Reakcja typowa leukocytów w wyniku oznaczania białek kationowych u dzieci z przewlekłym obustronnym ubytkiem słuchu (A) i w grupie kontrolnej dzieci zdrowych (B)

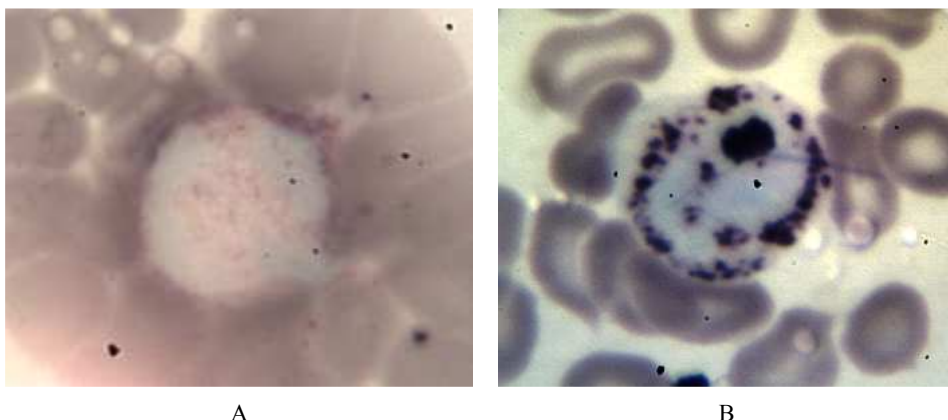


Fig. 5. Typical reaction to the detection of myeloperoxidase in peripheral blood granulocytes of the children with SNHL (A) and the children of the control group (B)

Ryc. 5. Reakcja typowa granulocytów w wyniku oznaczania mieloperoksydazy u dzieci z przewlekłym obustronnym ubytkiem słuchu (A) i w grupie kontrolnej dzieci zdrowych (B)

latter is chloride, which is oxidized initially to hypochlorous acid, with the subsequent formation of chlorine and chloramines. These products of the MPO-H₂O₂-chloride system are powerful oxidants that can have profound biological effects (Klebanoff 1999). From another hand, these same toxic agents can be released to the outside of the cell, where they may attack normal tissue and thus contribute to the pathogenesis of disease (Klebanoff 2005). The primary function of neutrophils is the phagocytosis and destruction of microorganisms, and the release of MPO and H₂O₂ into the phagosome containing the ingested microorganism generally leads to a rapid microbicidal effect (Klebanoff 1999). Prokopowicz et al. (2012) suggest that the pro-inflammatory pathological role of MPO may be a particular feature of chronic inflammation. In the physiological setting of acute neutrophil-mediated inflammation MPO may also form part of a negative feedback loop which down-regulates inflammation, limits tissue damage, and facilitates the switch from innate to adaptive immunity (Prokopowicz et al. 2012). In our study, the MPO level was also lower in the granulocytes of the children with SNHL compared to healthy children (0.65U vs. 2.2U) (Fig. 5).

Kinoshita and Tomiyama (1994) reported about immune reactions in the endolymphatic sac of guinea pigs caused spontaneous nystagmus, a suppressed caloric response, and fluctuating hearing loss. They result indicated that free radicals in the MPO-H₂O₂-chloride system were responsible for the tissue damage and dysfunction resulting from inner ear immune reactions. Myeloperoxidase staining can be seen in the endolymphatic sac, outer hair cells, stria vascularis and lateral canal (ampulla), and its time course parallels the time course of neutrophil infiltration of the endolymphatic sac (Kinoshita, Tomiyama 1994).

Phospholipids are important biological compounds found in the membranes of organelles (e.g mitochondria) of cells, including plasma, mitochondrial, chloroplast and bacterial membranes (Gevorkian et al. 1984). Phospholipids are of critical importance in cell biology, both through providing a permeability barrier and acting as

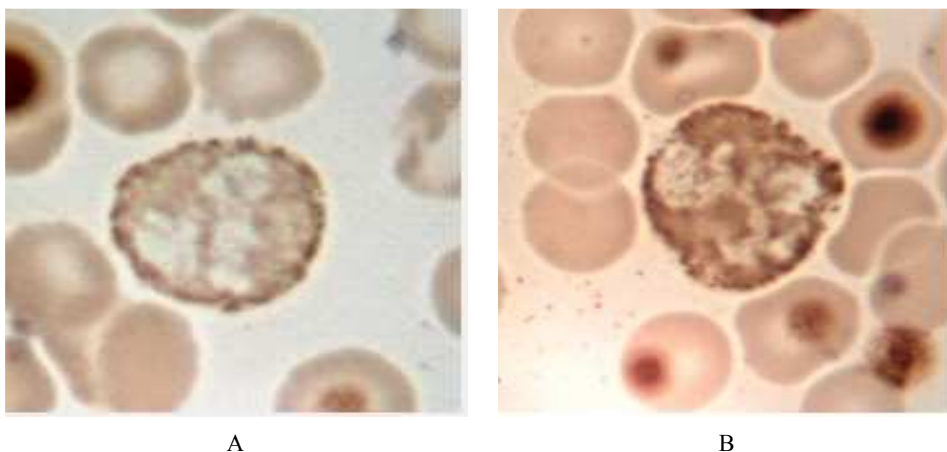


Fig. 6. Typical reaction to the detection of phospholipids in peripheral blood granulocytes of the children with SNHL (A) and the children of the control group (B)

Ryc. 6. Reakcja typowa granulocytów w wyniku oznaczania fosfolipidów u dzieci z przewlekłym obustronnym ubytkiem słuchu (A) i w grupie kontrolnej dzieci zdrowych (B)

substrates for synthesis of lipid mediators. O'Donnel and Murphy (2012) showed that phospholipid oxidation by acutely activated immune cells is a controlled event, and participate a central role in regulating membrane biology and innate immune function during health and disease. Recently, several new families of bioactive lipids were identified that form through the enzymatic oxidation of membrane phospholipids in circulating innate immune cells and platelets. These comprise eicosanoids attached to phosphatidylethanolamine and phosphatidylcholine and form within 2-5 minutes of cell activation by pathophysiologic agonists, via the coordinated action of receptors and enzymes (O'Donnel, Murphy 2012). Our study suggest, that children with SNHL possessed a decreased level of phospholipids (2.1 units) compared to the control group (2.3 units) (Fig. 6, 7D).

Alkaline phosphatase (ALP) refers to a group of phosphomonoesterases that hydrolyze phosphate esters with optimum in vitro activity at a pH of 10, resulting in the formation of an organic radical and inorganic phosphate. In mammals, this enzyme is found mainly in the liver and bones. Marked increase in serum ALP levels, a disease known as hyperalkalinephosphatasemia, has been associated with malignant biliary obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic lymphoma and sarcoidosis (Vroon, Israili 1990). In our study, the median of alkaline phosphatase activity in the children with SNHL was 2.3 units. The healthy children had 0.27 units (Fig. 7A).

The most prominent feature of the eosinophils are their large secondary granules, each containing four basic proteins, the best known being the eosinophil cationic protein (ECP). This protein has been developed as a marker for eosinophilic disease (Bystrom et al. 2011). The ECP is a secretory ribonuclease, which is found in the eosinophilic leukocyte and involved in the innate immune system. Its cytotoxic activity is effective against a wide range of pathogens, suggesting a relatively non-specific mechanism of action (Boix et al. 2008). Elevated ECP levels are found in

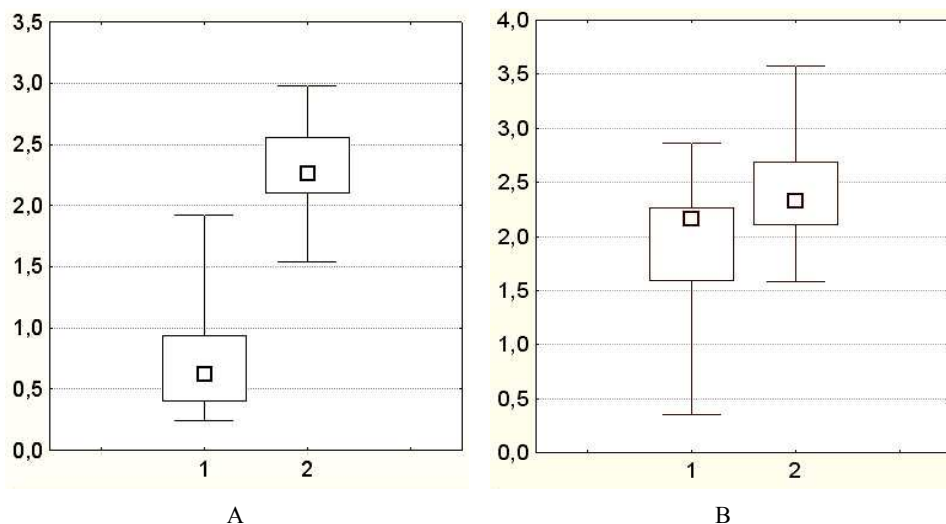
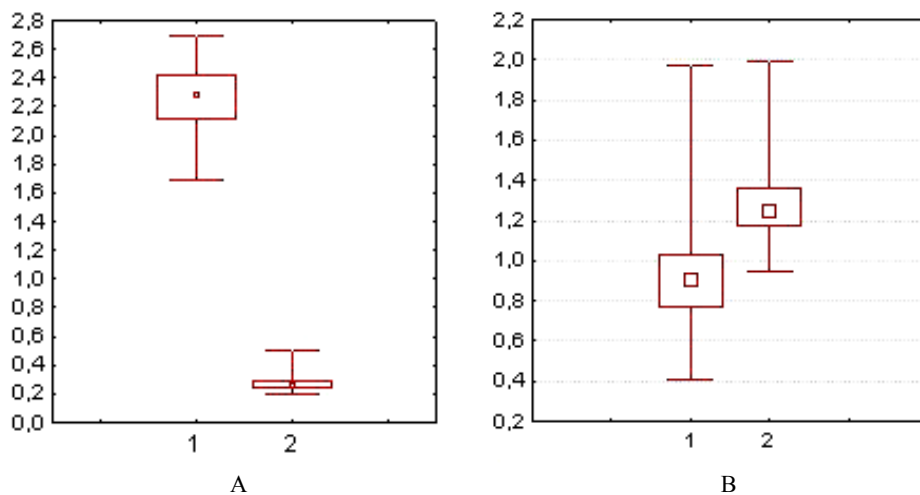


Fig. 7. Medians of granulocytes' metabolic activity in the group of the children with SNHL (1) and the children of the control group (2) ($p \leq 0.05$) (A – alkaline phosphatase, B – cationic protein, C – myeloperoxidase, D – phospholipids)

Ryc. 7. Mediana metabolicznej aktywności granulocytów u dzieci z przewlekłym obustronnym ubytkiem słuchu (1) i grupy kontrolnej dzieci zdrowych (2) ($p \leq 0,05$) (A – fosfataza zasadowa, B – białko kationowe, C – mieloperoksydaza, D – fosfolipidy)

T helper lymphocyte type 2 (atopic) diseases. ECP is a ribonuclease which has been attributed with cytotoxic, neurotoxic, fibrosis promoting and immune-regulatory functions. ECP regulates mucosal and immune cells and may directly act against helminth, bacterial and viral infections (Bystrom et al. 2011). The level of CP measured in the blood of the children with SNHL was higher compared to healthy children (Fig. 7B).

MPO is a major protein constituent of the primary granules of vertebrate neutrophils. It catalyses the H_2O_2 -mediated oxidation of halide ions to hypohalous acids, especially HOCl. These reactive oxygen species can participate in a variety of secondary reactions, leading to modifications of amino acids and many types of biological macromolecules (Prokopowicz et al. 2012). The granule enzyme MPO plays an important role in neutrophil antimicrobial responses (Metzler et al. 2011). Neutrophils release decondensed chromatin termed neutrophil extracellular traps to trap and kill pathogens extracellularly. Reactive oxygen species are required to initiate this formation but the downstream molecular mechanism is unknown (Papayannopoulos et al. 2010). Papayannopoulos et al. (2010) show that upon activation, escapes from azurophilic granules and translocates to the nucleus, where it partially degrades specific histones, promoting chromatin decondensation. Subsequently, MPO synergizes with neutrophil elastase in driving chromatin decondensation independent of its enzymatic activity. Here we show that neutrophils' myeloperoxidase from children with SNHL was lower compared to the children from control group (Fig. 7C).

It is known that inflammation of the inner ear in children, sinus contents and blood levels of IgG increased. The rate of increase in IgG correlates with disease severity. IgA and IgM levels, which are responsible for the primary immune response, almost unchanged (Veldman 1998).

It is known that interleukins regulate immune response. The ratio of anti-inflammatory interleukins and determines the type of immune response: cellular or humoral immune response. The main role of IL-2 – the transformation is not specific helper T cells in T-helper cells of the first type and the prevalence of cellular immune responses. The main role of IL-4, IL-5, IL-10, IL-13 is to direct the immune response to “humoral” type, by activation of B lymphocytes (Broughton et al. 2004).

Antibodies are a vital part of the armamentarium of the adaptive immune system for the fine-tuning of the recognition and response to foreign threats (Grönwall et al. 2012). Antibodies function in a variety of ways designed to eliminate the antigen that elicited their production. Immunoglobulin A (IgA) is found in high concentrations in the mucous membranes, particularly those lining the respiratory passages and gastrointestinal tract, as well as in saliva and tears. Immunoglobulin G (IgG), the most abundant type of antibody, is found in all body fluids and protects against bacterial and viral infections. Immunoglobulin M (IgM), which is found mainly in the blood and lymph fluid, is the first to be made by the body to fight a new infection. For example, only IgG and IgM antibodies have the ability to interact with and initiate the complement cascade. Likewise, only IgG molecules can bind to the surface of macrophages via Fc receptors to promote and enhance phagocytosis. IgA, IgG, and IgM are frequently measured simultaneously. Evaluated together, they can give doctors important information about immune system functioning, especially relating to infection or autoimmune disease. IgA antibodies have potent immunomodulatory properties, being able to both induce and suppress immune responses. IgA-mediated inhibitory function is able to inhibit several inflammatory diseases including asthma and glomerulonephritis (Schwartz-Albiez et al. 2009). Determining the immunoglobulin's levels in serum and saliva are presented in Table 1. These results show a few significant ($p \leq 0.05$) differences between group of the children with SNHL and group of healthy children (Table 1).

Table 1

Immunoglobulin's level in the blood and saliva in studied children (g per L), M±m

Tabela 1

Poziom immunoglobulinów we krwi i ślinie badanych grup dzieci (g/L), M±m

Group		IgA	IgM	IgG
Children with SNHL (n=60)	blood	2.5 ± 0.043	0.9 ± 0.016	21.5 ± 0.303
	saliva	0.3 ± 0.008	0.5 ± 0.014	1.96 ± 0.076
Healthy children (n=60)	blood	1.5 ± 0.025*	1.3 ± 0.015*	12.01 ± 0.21*
	saliva	1.3 ± 0.014*	0.8 ± 0.018*	1.0 ± 0.021*

* Statistically significant difference between the studied groups (p≤0.05)

Decreased salivary immunoglobulin A (sIgA), a component of mucosal immunity, is associated with SNHL. We investigated significant changes in sIgA in the children with SNHL. Salivary IgA level in the children with SNHL was lower (0.3 ± 0.008) g/L compared to the controls (1.3±0.014) g/L. While the IgA level in the blood of the children with SNHL was higher (2.5±0.043) g/L compared to the controls (1.5±0.025) g/L. Our results are in agreement with reports of other authors, which suggest that SNHL can induce immune reactions by increasing the formation of immunoglobulins. For example, Dünne et al. (2004) evaluated a possible role of an endured infection with *Chlamydia pneumoniae* or *Chlamydia trachomatis* as a cause for sudden sensorineural hearing loss. For this in 60 patients with a first episode of a SNHL and 60 sex-matched and aged-matched control, following complete otoneurological diagnosis blood tests for IgA, IgM and IgG with regard to *Chlamydia pneumoniae* and trachomatis were evaluated. They found a statistically significant higher prevalence of IgA positivity of *Chlamydia pneumoniae* in patients with sudden SNHL (Dünne et al. 2004).

IgG are the most common isotype of immunoglobulins and include four subclasses which differ from one another in the following ways: their initial amino acid sequence, their physical and chemical properties and the different serum concentrations reached with age. Every subclass has a specific biological function: the response to proteic antigens is prevalently mediated by IgG1 and IgG3, while IgG2 mediates the response to polysaccharide antigens. Although low levels of IgG subclasses may be temporary, deficiencies are often associated with various diseases: 1) recidivating bacterial infections involving the respiratory and digestive tracts, primarily sustained by capsulated or pyogenic microorganisms; 2) IgA deficiency; 3) absence of immune response following vaccination; 4) allergic or autoimmune diseases; 5) diseases of the CNS (Cataldo, Paternostro 1990). In our study, the IgG level in the blood of the children with SNHL was higher compared to the children with a control group. Moreover, the IgG level in the saliva of the children with SNHL was also higher than in the healthy children (Table 1).

Serum IgM exists as a pentamer in mammals, predominates in primary immune responses to most antigens, is the most efficient complement fixing immunoglobulin and comprises approximately 10% of normal human serum Ig content. IgM is also

expressed on the plasma membrane of the B lymphocytes as a monomer. It is the B cell antigen receptor and the H chains each contain an additional hydrophobic domain for anchoring in the membrane. Monomers of serum IgM are bound together by disulfide bonds and a joining (J) chain (Davis, Shulman 1989). Autoantibodies of the IgM type, on the other hand, have shown promising results in the treatment of multiple sclerosis. These autoantibodies promote remyelination rather than modulating inflammation. Oxidation-specific epitopes, as found in atherosclerotic lesions and on apoptotic cells, comprise one important target of natural antibodies. By recognizing these epitopes, natural antibodies neutralize proinflammatory responses and mediate atheroprotection (Schwartz-Albiez et al. 2009). In our study, the mean concentration of IgM was lower in the blood of children with SNHL compared with the control group. The saliva IgM level was also lower in the children with SNHL (Table 1).

Recent evidence is discussed that NE and epinephrine, through stimulation of the beta(2)-adrenoreceptor-cAMP-protein kinase A pathway, inhibit the production of type 1/proinflammatory cytokines, such as interleukin (IL-12), tumor necrosis factor-alpha, and interferon-gamma by antigen-presenting cells and T helper (Th) 1 cells, whereas they stimulate the production of type 2/anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta. Through this mechanism, systemically, endogenous catecholamines may cause a selective suppression of Th1 responses and cellular immunity, and a Th2 shift toward dominance of humoral immunity. On the other hand, in certain local responses, and under certain conditions, catecholamines may actually boost regional immune responses, through induction of IL-1, tumor necrosis factor-alpha, and primarily IL-8 production (Elenkov et al. 2000).

Cytokines represent the major factors involved in the communication between T cells, macrophages and other immune cells in the course of an immune response to antigens and infectious agents (Belardelli 1995). Much data support an essential role for interleukin IL 2 in immune tolerance. IL-2 sometimes contributes to optimal primary immune responses, but it is not mandatory. Emerging findings, however, suggest an essential role for IL-2 in immune memory (Malek 2008). In our study, the IL-2 level was lower in the children with SNHL (2.3 ± 0.05) pg/mL compared to the controls (3.6 ± 0.03) pg/mL (Table 2).

Table 2
Interleukins level in the blood and saliva the studied groups of children (pg per mL), M \pm m
Tabela 2
Poziom interleukinów we krwi i ślinie badanych grup dzieci (pg/mL), M \pm m

Group		IL-2	IL-4	IL-5	IL-10	IL-13
Children with SNHL (n=60)	blood	2.3 ± 0.05	11.02 ± 0.12	5.6 ± 0.03	8.8 ± 0.05	29.9 ± 0.66
	saliva	0.04 ± 0.01	54.2 ± 0.13	3.8 ± 0.04	0.9 ± 0.05	19.4 ± 0.63
Healthy children (n=60)	blood	$3.6 \pm 0.03^*$	$8.04 \pm 0.01^*$	$3.7 \pm 0.02^*$	$2.2 \pm 0.023^*$	$8.01 \pm 0.01^*$
	saliva	$1.2 \pm 0.02^*$	$0.3 \pm 0.01^*$	$1.03 \pm 0.02^*$	$0.03 \pm 0.004^*$	$2.3 \pm 0.01^*$

* Statistically significant difference between the studied groups ($p \leq 0.05$)

Interleukin-4 is a cytokine that regulates multiple biological functions. It can regulate proliferation, differentiation, and apoptosis in several cell types of haematopoietic and non-haematopoietic origin. It has a critical role in the regulation of Th0 cell differentiation during a normal immune response, and IL-4-driven Th2 cells direct host responses against parasitic infections. The IL-4 and its signalling machinery have been involved in the development of immune diseases including allergy, autoimmunity and cancer (Zamorano et al. 2003). The IL-4 level in the blood of the children with SNHL was higher (11.02 ± 0.12) pg/mL than in the controls (8.04 ± 0.01). The salivary IL-4 level was elevated (54.2 ± 0.13) pg/mL vs. with controls (0.3 ± 0.01) pg/mL (Table 2).

Interleukin 5 (IL-5) has been shown to play an instrumental role in eosinophilic inflammation in allergic diseases (Corren 2011). Elevated numbers of blood and tissue eosinophils are present in allergic diseases and experimental evidence suggests that eosinophils play an important pathogenic role in these conditions. Regulation of eosinophil maturation, recruitment, and survival is under the control of a small

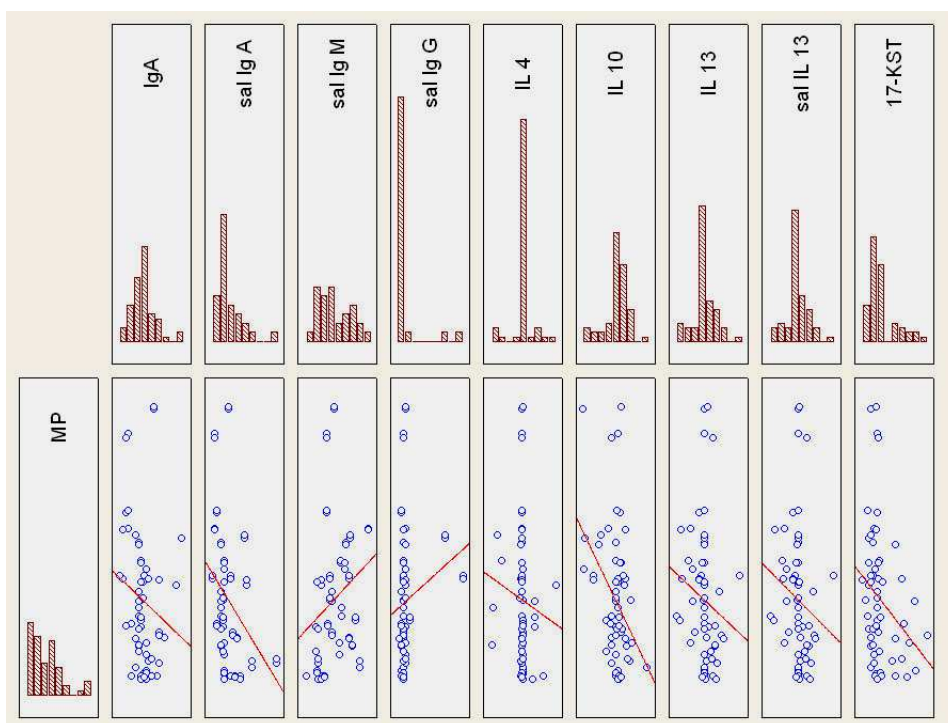


Fig. 8. Correlations analysis between myeloperoxidase (MP) activity and IgA level in the blood of the children with SNHL (n = 60); IgA, IgM, IgG in the saliva (sal IgA, sal IgM, sal IgG); interleukins (IL-4, IL-10, IL-13), interleukin-13 in saliva (sal IL-13), 17-ketosteroids (17-KST)

Ryc. 8. Analiza korelacyjna między aktywnością mieloperoksydazy (MP) i poziomem immunoglobuliny IgA we krwi dzieci z przewlekłym obustronnym ubytkiem słuchu; IgA, IgM, IgG w ślinie (sal IgA, sal IgM, sal IgG); interleukinami (IL-4, IL-10, IL-13), interleukinem-13 w ślinie (sal IL-13), 17-ketosteroidami (17-KST)

group of factors, including IL-5 (Corren 2012). Interleukin-10 (IL-10), first recognized for its ability to inhibit activation and effector function of T cells, monocytes, and macrophages, is a multifunctional cytokine with diverse effects on most hemopoietic cell types (Moore et al. 2001). IL-10 is an important suppressive cytokine, produced by a large number of immune cells in addition to the antigen-driven IL-10-producing regulatory and the naturally occurring suppressor CD4+ T cells, which is a key player in anti-inflammatory immune responses (O'Garra et al. 2004). In addition to these activities, IL-10 regulates growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells. IL-10 plays a key role in differentiation and function of a newly appreciated type of T cell, the T regulatory cell, which may figure prominently in control of immune responses and tolerance in vivo (Moore et al. 2001). Interleukin (IL)-13 plays a major role in various inflammatory diseases including cancer, asthma, and allergy. It mediates a variety of different effects on various cell types including B cells, monocytes, natural killer cells, endothelial cells, and fibroblasts (Joshi et al. 2006). In our study, the children with SNHL possess high levels of IL-5, IL-10 and IL-13, both in the blood and saliva (Table 2).

We also studied the correlations between the indicators. There was found 51 relationships between the data in the group of the healthy children, as well as 53 relationships in the group of the children with SNHL (Fig. 8). Interestingly, correlations between the indicators in the group of the children with SNHL were distributed unequally. Many significant correlations was observed in the granulocyte myeloperoxidase activity ($n = 9$) (Fig. 8).

Direct correlation was observed between the MPO activity and IgM level ($r = 0.37$, $p \leq 0.05$), MPO activity and IgG level ($r = 0.26$, $p \leq 0.05$). The inverse cor-

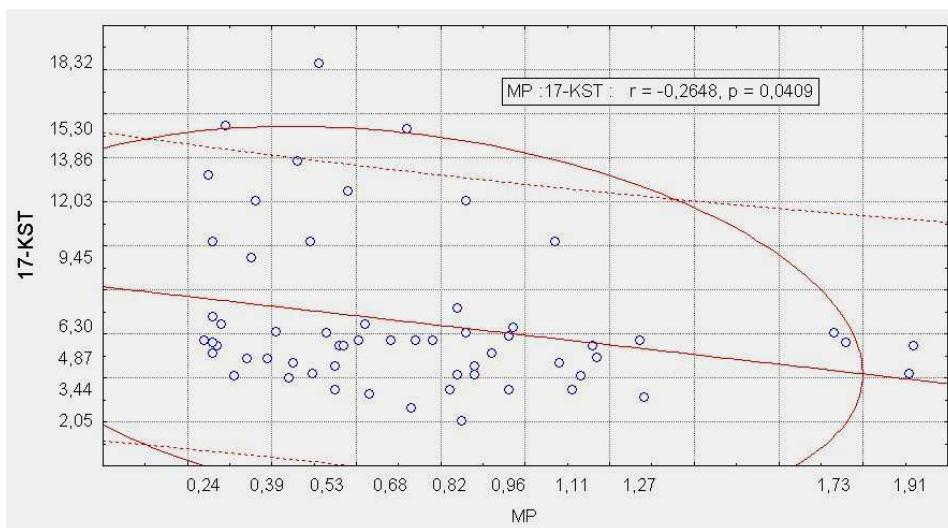


Fig. 9. Correlation between myeloperoxidase (MPO) activity in granulocytes and 17-ketosteroids (17-KST) level

Ryc. 9. Analiza korelacyjna między aktywnością mieloperoksydazy (MPO) w granulocytach i poziomem 17-ketosteroidów

relation between MPO activity and IgA level ($r = -0.29$, $p \leq 0.05$), as well as MPO activity and salivary IgA level ($r = -0.42$, $p \leq 0.05$) was noted. The inverse correlation between MPO activity and both blood IL-10 and IL-13 levels ($r = -0.27$, $p \leq 0.05$), and salivary IL-13 level ($r = -0.29$, $p \leq 0.05$) also was studied. Correlation between MPO activity and 17-ketosteroids was also inverse ($r = -0.29$, $p \leq 0.05$) (Fig. 9).

The brain and the immune system are the two major adaptive systems of the body. During an immune response the brain and the immune system “talk to each other” and this process is essential for maintaining homeostasis. Two major pathway systems are involved in this cross-talk: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). It is known that stress hormones affect T-helper cells activity, and cytokine levels in the blood (Elenkov et al. 2000). Long-term stress hormones impact causes hyperactive states in the white blood cells. Alkaline phosphatase activity is increased in the hyperactive leukocytes. On the other hand, the myeloperoxidase level, cationic proteins and lipids level are decreases.

Active leukocytes produce increased level of IL-4, IL-5, IL-10, and IL-13. Stress hormones and IL-4 affect the maturation of T-helper cells. “Naive” T-helper cells are transformed into T-helper cells of the second type (Th2). Stress hormones and IL-4 suppress the production of IL-2. At the same time, IL-4, IL-5, IL-10, IL-13, and Th2 co-operate with B-lymphocytes. B-cells become hyperactive and turn into plasma cells. B-cells produce many antibodies in the blood of the children with hearing loss. Our results suggest that there is an imbalance between immunoglobulins’ levels in the blood and saliva. This phenomenon in the literature named as “the effect of delayed stress” (McClelland et al. 1980, Evans et al. 1994, Tsujita, Morimoto 1999). The activation of sympathetic nervous system during an immune response might be aimed to localize the inflammatory response, through induction of neutrophil accumulation and stimulation of more specific humoral immune responses, although systemically it may suppress Th1 responses, and, thus protect the organism from the detrimental effects of proinflammatory cytokines and other products of activated macrophages (Elenkov et al. 2000).

CONCLUSIONS

Our results suggest that the children of 7-11 years old with SNHL possess increased urine adrenaline and 17 ketosteroids levels. This indicates that the stress reaction is occurs. Elevated hormones levels lead to changes in the immune system activity. Adrenaline and 17-ketosteroids activated leukocytes. The increasing of alkaline phosphatase activity and decreasing myeloperoxidase activity, as well as cationic proteins and lipids take place in leukocytes. Activated leukocytes release anti-inflammatory interleukins. B-lymphocytes are activated and transform into plasma cells. These interleukins (especially IL-4) are affecting the maturation of Th2. They also stimulate B cells to provide an immunoglobulins release. We observed elevated blood levels of IgA and IgG, and decreased IgM level in the group of the children with SNHL. High IgG level and decreased IgA and IgM levels were observed in saliva of the children with SNHL. Impairment of immunoglobulins levels, as well as

metabolic changes of leukocytes leads to reduced non-specific immunity in the children with SNHL.

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