SOME ASPECTS OF THE PERINATAL WATER AND ELECTROLYTE METABOLISM AND EXAMINATION OF THE UROGENITAL TRACT OF THE NEWBORNS. CLINICAL OBSERVATIONS AND ANIMAL EXPERIMENTS.

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PhD thesis

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1. Introduction

In addition to the successful ambition of the obstetricians to minimize the frequency of premature births, the efforts made by the pediatricians for a more comprehensive understanding of physiologic features of the neonates, unraveling the processes, preventing and treating of the pathologic conditions of the newborns play a pivotal role in improving postnatal mortality. These efforts resulted in a remarkable development in the management of the very low birth weight premature infants.

The investigation of the regulation of the neonatal acid-base and electrolyte homeostasis has a great tradition in the Medical University of Pécs. As a result of these studies, the most important components of the renal and endocrine regulation of electrolyte handling, the peculiar relationship between the acid-base and electrolyte homeostasis and the maturational changes were revealed.

These studies demonstrated, that the mature neonates even under physiologic circumstances develop renal salt wasting, and consequently a negative sodium balance, referred as late hyponatraemia occur. In background of this late hyponatraemia and metabolic acidosis, an insufficient renal H+-Na+ exchange was proven and it was first established, that sodium supplementation prevents or rather corrects the transient disturbance of electrolyte homeostasis, that indicates a marginal somatic instability of the premature infants.

2. Objectives

The aims of our examinations, summarized in the dissertation, were to clear up the role of the ouabain-like substance in the electrolyte handling during the neonatal period, the perinatal alterations of the physical water compartments of the brain and the lung and the relationship between the renal AQP2-excretion and the concentrating capacity of the kidney. We paid a specific attention to ascertain the functions those autocrine/paracrine acting vasoactive substances normally expressed in the ureter (endothelin-1, adrenomedullin, iso-enzymes of nitric oxyde synthase, neuropeptide-Y) in the pathogenesis of the uretero-pelvic obstruction. Our investigations were carried out in frame of wide national and international cooperation applying modern laboratory methods (real-time quantitative PCR, high-performance liquid chromatography, radio immunoassay, H_1-NMR spectroscopy).

Our results can contribute to a more comprehensive cognition of the perinatal physiologic and pathophysiologic processes and support an introduction of more effective treatment. The quantitative analysis of the vasoactive hormones in the ureter can provide useful information on the mechanism of the developmental disorders of the urogenital tract and their future therapy.

3. Materials and methods

Urinary excretion of endogenous ouabain-like substance in NaCl supplemented prematures

Two groups of healthy, premature male infants appropriate in size for gestational age were studied. Group S consisted of nine infants with birth weights of 1280 to 1750 g and gestational ages of 29 to 33 weeks. These infants were given sodium supplementation in a dose of 3 to 5 and 1,5 to 2,5 mmol/kg per day at a postnatal age of 8 to21 and 22 to 35 days, respectively. Group NS, that included nine infants with birth weights ranging from 125 to 1810 g and gestational ages of 28 28 to 34 weeks, received no salt supplements. All infants were born vaginally after an uncomplicated pregnancy and no signs and laboratory evidence of perinatal asphyxia, infection or cardiopulmonary distress were noted thereafter. Plasma sodium concentration and daily excretion of sodium, potassium, creatinine (modified Jaffé-reaction) and ouabain (RIA, HPLC) were determined from urine collected for 24 hours on the seventh day of life and at weekly intervals up to the fifth week. Statistical analysis was done by paired t-test. Informed parental consent and approval of the institutional ethics committee were obtained for the study.

Examination of lung and brain water by proton magnetic relaxation in preterm and term rabbit pups

Examination were performed in fetal Pannon white rabbit pups at gestational ages of 25 (n=17), 27 (n=14), 29 (n=17) and 31 days (n=12), born by elective cesarean section under epidural anaesthesia. An additional group of 18 full-term (n=31) newborn rabbits was studied at a postnatal age of 4 days.

Immediately after delivery, the animals were killed by an overdose of sc. phenobarbital and the lung and brain were removed as quickly as possible for determination the tissue water content, NMR relaxation times and HA concentration. NMR measurement was made promptly after obtaining the specimens, the rest of the samples were weighed and frozen and then freeze-dried for 72 h. The dried tissue specimens were reweighed, and the water content was calculated as a wet to dry weight ratio. The dried tissue was then digested with pronase and the HA content was determined with a radiometric assay kit.

Tissue samples of approximately 200 mg were placed in 5-mm-diameter NMR glass tubes and incubated at 40 °C for 5 min. MRS was performed on a Bruker Minispec PC 140 portable MR spectroscope. T_1 relaxation time was measured by an inverse recovery method with eight different time intervals between 180° and 90° pulses. T_2 relaxation time was obtained by using the Carr-Purcell-Meiboom-Gill sequence. If the tissue specimen consist of independent water compartment

characterized by different relaxation rates, this function can be expressed by multiexponential equation. For the determination of the free and bound water compartments, characterized by T_{21} and T_{22} relaxation curves, respectively, biexponential fitting was applied.

Gene expression analysis of ureter sections from pyelo-ureteral stenosis

Stenotic ureteral tissue sections from eight children (mean age: 4,1 ys, ranging from 6 weeks to 12 ys) with pyelo-ureteral obstruction operated according to Anderson-Hynes were studied. Diagnosis was based upon radiologic and scintigraphic findings. Ureter specimens from adult patient undergone tumor nephrectomy or bladder tumor resection (n: 7, mean age: 73 ys, ranging from 56 to 84 ys) served as control. The samples were immediately frozen after removal and kept at -70 C. Total RNA was isolated from the tissue specimens by RNA-zol kit, and the RNA content was measured by spectrofotography. 2 pg of the RNA in 40 µl solution of each samples was reversely transcribed, and subjected to gene expression studies using quantitative real time PCR analysis (Perkin Elmer 7700). Messenger RNA expression of the neuropeptide-Y, endothelin-1, the recently discovered adrenomedullin and the different isoform of nitric oxyde synthase, the neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) were studied. As houesekeeping genes, glyceraldehid-3-phosphat (GAPDH), CD31, smooth muscle actin (Smactin) and protein gene product 9.5 served. A dilution sequence of the PCR product of the target gene was used as external reference in the PCR reaction. Oligonucleotides of each target gene were selected by the Primer Express (Perkin-Elmer) software, using the same PCR parameters.

Urinary excretion of aquaporin-2 in preterm and term infants

Five healthy premature infants (mean gestational age: 30,6 weeks, ranging from 30 to 32 weeks; mean birth weight: 1570 g, ranging from 1480g to 1670 g) and nine mature infants (mean gestational age: 39,2 weeks, ranging from 37 to 40 weeks; mean birth weight: 3218 g, ranging from 2760g to 4720 g) were enrolled. The determination of the gestational age was based on the maternal menstruation history and was confirmed by intrauterine ultrasonography as well as maturational signs (Dubowits score) after birth. Each infant was born vaginally from an uncomplicated pregnancy and had a birth weight between 10 and 90 percentile. The Apgar score was above 7 in the first minute and the perinatal period was uncomplicated in each case. The mature newborns were examined on the 1., 3., and 5. postnatal day, when they received 62 ml/kg, 86 ml/kg and 116 ml/kg daily fluid intake, respectively. The premature infants were studied on the 7. day and weekly thereafter up to 6. week of life. The infants received mother milk supplemented with 5% glucose

solution for reaching the daily fluid intake (158-195 ml/kg). Urine samples were collected 3-4 hours after the last feeding morning in each time-point for determination of osmolality, creatinine and AQP-2. Urinary AQP-2 was measured by high sensitivity radioimmunoassay.

4. Results

Urinary excretion of endogenous ouabain-like substance in NaCl supplemented prematures

In preterm infants on sodium intake urinary sodium excretion declined steadily during the first four week of life, negative sodium balance developed. Premature infants receiving supplemental sodium chloride had higher urinary sodium excretion, but retained more sodium, maintained plasma sodium concentration at normal level. The rate of urinary ouabain excretion was similar in the two groups prior to NaCl supplementation. In infants not receiving sodium supplementation ouabain excretion increased significantly from 180±9.7 pg/kg/h in the first week to 260±11 pg/kg/h in the third week ($p<0;01$) and remained at about the same level thereafter. NaCl supplementation induced a marked, although not significant decline in urinary ouabain excretion and no further consistent change occured during the course of the study. As a result, the differences in urinary ouabain excretion between the two groups proved to be significant at the level of $p<0,001$ during weeks 2 to 5. The pattern of postnatal changes in urinary ouabain excretion when expressed per mg creatinine appeared to be similar and its values reached again significantly higher levels in infants without NaCl supplementation than in those supplemented with NaCl in weeks 2 to 5.There was no relationship between urinary sodium and ouabain excretion in prematures on low sodium intake. In infants receiving high sodium diet, however, urinary ouabain was directly related to sodium excretion ($r=0.66$, $p<0,001$).

Examination of lung and brain water by proton magnetic relaxation in preterm and term rabbit pups

As the gestation advanced, brain water content expressed as wet-to-dry ratio decreased steadily at a slow rate from an initial value of 9.6±0.3 at 25 days to values of 9.3±0.2 at 27 days, 9.2±0.2 at 29 days and 9.1±0.3 on 31. day followed an abrupt fall to 8.7±0.3 at 4. day postnatally. The trends and time courses of T_1 and T_2 relaxation times proved to be quite similar. Interestingly, the postnatal fall of T_2 was preceded by a significant decrease on the 31. day. Biexponential analysis of the T_2 relaxation curves made it possible to distinguish the fast (T_{21}) and slow (T_{22}) components that represented the bound and free water fractions. It can be seen that T_{21} tended to increase progressively with gestational age from 102.4±40.6 ms on 25 days to 135.5±64.3 ms on 31 days after which it decreased to 92.6±28.8 ms postnatally. T_{22} rose between the 25 days and 29 days,

when it peaked and then declined steadily until the 4. postnatal day. As a result, the contribution of the bound water fraction represented by T_{21} amounted to only 4.4±1.7% of the total brain water at the gestational age of 25 days, reached its maximum of 14±17.6% at 29 days and then decreased gradually to 4.7±4.3% at 31 days of gestation and to 3.3±0.3% postnatally. While the total brain water decreased from the gestational age of 29 to 31 days by only 0.8%, the corresponding reduction in T_2 relaxation time was 4.6% and that in the bound water fraction derived from T_{21} was 65.4%. This findings are regarded as strong evidence for restructuring of fetal brain water as the gestation advances to term. It is demonstrated that the high hyaluronan concentration of 2095±273.4 µg/g dry weight at a gestational age of 25 days was markedly depressed by 27 days (1541±168.8 µg/g dry weight, $p<0.001$) and at 29 days (1527±316 µg/g dry weight, $p<0.001$) which was followed by a transient increase to 1826±292 µg/g dry weight at 31 days. Postnatally in rabbit pups born at term HA dropped to an extremely low level of 1144±126.3 µg/g dry weight ($p<0.001$) as compared with the fetal values.

Lung water expressed as wet-to-dry ratio did not change with advancing gestation from 25 days to 29 days, then it started to decline at 31. days, followed by a marked fall at 4 day postnatally. The trends and time courses of T_1 and T_2 relaxation times proved to be quite similar. The bound and free water fractions represented by the fast (T_{21}) and slow (T_{22}) components distinguished by biexponential analysis of the T_2 relaxation curves increased until 27. days, and then decreased progressively until the end of examination period. The relative contribution of the bound water fraction amounted to 31% to 34% of the total lung water in the period between 25 and 29 days of gestation, decreased significantly before delivery, and decreased even further until a postnatal age of 4 days after birth at terms. As gestation advanced, the HA concentration related to the dry tissue declined, after birth, however, there was a 2-fold increase in its concentration in spite of the concomitant abrupt fall in lung tissue water, especially in the bound fraction. The water content of the lung correlated positively with the T_1 ($p<0.001$, $r=0.87$) and T_2 ($p<0.001$, $r=0.93$) relaxation times as well as the bound water fraction ($p<0.001$, $r=0.67$). Hyaluronan content correlated only with the total tissue water content ($p<0.001$, $r=0.39$) and it proved to be independent from the T_1, T_2 and T_{21} relaxation times.

Gene expression analysis of ureter sections from pyelo-ureteral stenosis

We could demonstrate a significantly higher endothelin-1 mRNA expression related to CD31 as housekeeping gene and decreased adrenomedullin mRNA expression related to GAPDH in the stenotic ureter segments than in the control tissue group. No difference was noted in nNOS, eNOS, iNOS and neuropeptide-Y RNA expression between the two groups. NPY expression values proved

solution for reaching the daily fluid intake (158-195 ml/kg). Urine samples were collected 3-4 hours after the last feeding morning in each time-point for determination of osmolality, creatinine and AQP-2. Urinary AQP-2 was measured by high sensitivity radioimmunoassay.

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Gene expression analysis of ureter sections from pyelo-ureteral stenosis

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to be much lower compared to the other nitrerg, peptiderg substances studied.

Urinary excretion of aquaporin-2 in preterm and term infants

Urinary AQP-2 concentration in mature infants was the highest on the 1. postnatal day (74±20 nmol/µl) and fell to 30±12 nmol/µl and 44±13 nmol/µl on day 3 and 5, respectively. Basically similar pattern in urinary creatinine excretion had been observed, however, more prominent drop occured (470±83 mg/l and 132±8 mg/l on the 1. and 5. day, respectively). As a result, AQP-2 excretions expressed per mg creatinine were 194±45 fmol/mgCr, 121±36 fmol/mgCr and 322±90 fmol/mgCr in the above mentioned time-points. In contrast, an opposite trend had been observed in urinary osmolality that showed a steadily decline with the advancing of the postnatal age.

5. Discussion

The study on ouabain excretion of premature infants kept on different sodium intake was delineated in the first part of the dissertation. We demonstrated that the urinary ouabain is significantly lower in NaCl supplemented prematures than that in the controls. Our results supports those observations, which found no direct relationship between the volume expansion and adrenal ouabain production. Concordantly with previous experimental observations, we firstly suggested, that the EDLF production and secretion in prematures regulated by the renin-angiotensin-II system. The demonstrated positive correlation between sodium and ouabain excretion refers to the role of EDLF in elimination of the excessive salt intake.

The better understanding of the peculiarities of the perinatal water handling necessitates the molecular level knowledge of the behaviour of the water. Our work group investigated the water content and the water fractions with different motility distinguished by H1-NMR spectroscopy in the brain and lung, as well as the maturational changes of the water binding hyaluronan during gestation and postnatally. According to our results, the fetal brain tissue is characterized by elevated hyaluronan and water content, with the predominance of the free motility fraction. The decline of these parameters occurs at the end of gestation and continue postnatally and proved to be independent from the bound water fractions, that let us to conclude, that the hyaluronan plays insignificant role in the realignment of the water compartments.

The examination of the lung tissue confirms the previous data, that the removal of the water from the lung in favor of better postnatal gas change begins prior birth. Additionally, our results provide evidence to the physical nature of the lung water, demonstrating a parallel decrease of the bound water and an increase in the motionally free water fraction. Similarly to the brain tissue, the bound water fraction proved to be independent from the hyaluronan content in the lung.

The results of the study on the aquaporin-2 excretion of mature and premature infants supports the previous data, that urinary AQP-2 concentration is low end its excretion undergo considerable changes during maturation. The AQP-2 excretion does not differ significantly between premature and mature infants in the first week of life referring to postnatal factors of maturation of intracellular transport and synthesis of AQP-2 regulated by arginino-vasopressin. Our data show the dissociation of renal AQP-2 and concentrating capacity suggesting asynchronous maturation of factors independent from AVP-AQP-2 axis.

Several paracrine/autocrine acting hormones, peptides and enzymes catalyzing their production with uncleared function can be detected in urogenital tract. Our molecular genetic examination on ureteric tissue samples from congenital pyelo-ureteral stenosis allows insights into some pathophysiologic processes. The elevated endothelin-1 and decreased adrenomedullin expression,

considering their physiologic effects, correlate with microanatomic alterations observed in the affected stenotic tissue segments.

Summary

1. The urinary ouabain excretion is diminished in NaCl supplemented premature infants. The correlation of the sodium and ouabain excretion suggests the possible role of ouabain in excessive sodium elimination.
2. The recompartmentalization of the water in the brain and the lung begins prior birth and the hyaluronan has no effect on this process.
3. The renal AQP-2 is independent from the concentrating capacity of the kidney in premature and mature infants.
4. Increased expression of endothelin-1 and decreased expression of adrenomedullin are observed in congenital uretero-pelvic obstruction.

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