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Department of Paediatrics

Specific properties of mitochondrial energy generating system in premature neonates

PhD Thesis summary

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1. INTRODUCTION

Most diseases in premature neonates are secondary to immaturity of various organ systems. Postnatal switch of glycolytic to oxidative metabolism is of crucial importance for all mammalian neonates and is essential for successful adaptation to extrauterine life. The recruitment of oxidative phosphorylation in the tissues of the neonates reflects changes in the hormonal status and a postnatal shift from glucose to glucose and fat as the major energy sources (Valcarce *et al.,* 1994).

More than 90% of ATP in mammalian cells is produced by oxidative phosphorylation system (OXPHOS) composed of four respiratory chain complexes (RC) and ATP synthase in the inner mitochondrial membrane. ATP synthase utilize energy of proton electrochemical gradient generated by respiratory chain complexes during substrate oxidation. The OXPHOS biosynthesis and the cellular capacity for energy production relies on proper assembly of >80 different protein subunits encoded by nuclear DNA (nDNA) as well as mitochondrial DNA (mtDNA). This process is coordinated by large amount of nuclear encoded proteins (assembly factors, chaperons, translocators across mitochondrial membranes, transcription factors controlling mtDNA and nDNA gene expression), but the proper mechanism of OXPHOS biogenesis and functional capacity in fetal and early postnatal period is still not fully understood. Both inherited and acquired OXPHOS disturbances represent a heterogeneous group of disorders, which may manifest early after birth with functional impairment of tissues with high energetic demands (brain, heart, muscle, liver).

Premature neonates represent the major contributors to neonatal morbidity. The inadequate capacity of mitochondrial energy production in cells due to delayed maturation of respiratory chain complexes or increased degradation of mitochondrial proteins due to acquired factors including infection or hypoxemia may represent additional risk for premature neonates.

It may be difficult, especially in critically ill neonates, to distinguish properly between the primary genetically encoded disorders of energy provision with mendelian and maternal inheritance and the secondary mitochondrial disturbances resulting in similar clinical phenotype (Smeitink *et al.,* 2003). The low activity of pyruvate dehydrogenase complex (PDH) in premature or critically ill neonates may not only negatively influence the ATP production but it may also be the cause of low glucose tolerance resulting in lactic acidosis (Vary, 1996). Only precise diagnostics on biochemical and molecular levels may be used for the correct genetic counseling in families with deceased child in early neonatal period.

Biochemical and molecular biology studies of the biogenesis of mitochondrial energy-converting system in perinatal period as well as analysis of functional consequences of delayed maturation of the mitochondrial OXPHOS apparatus and PDH with respect to postnatal adaptation and neonatal morbidity address highly actual biomedical problems. Better understanding of mechanism involved in mitochondrial biosynthesis and degradation is required to further improve the morbidity of premature neonates and their outcome.

2. AIMS OF THE STUDY

The inadequate capacity of energy production in early development may play an important role in the neonatal morbidity. The lower functional capacity of energy production in neonates may result from delayed maturation of mitochondrial complexes combined with increased degradation of mitochondrial proteins during infection.

The thesis is based experimentally on biochemical analyses in platelets and lymphocytes from umbilical cord blood, placental tissue obtained after the delivery of neonates in various gestational age and samples of muscle tissue from miscarriages obtained within an hour after the delivery and from autopsy obtained 1-2 hours after death of premature neonates.

The specific aim of this study has been:

a) to characterize the amount, composition and activities of respiratory chain complexes and pyruvate dehydrogenase in muscle tissue and isolated muscle mitochondria obtained at autopsy from miscarriages and critically ill neonates who died in neonatal period,
b) to analyse specific properties of heavy fraction of mitochondria from human-term placenta and glycerophosphate-dependent hydrogen peroxide production,
c) to evaluate free carnitine and total carnitines levels in cord blood and in blood obtained during the first days of life in neonates born in various gestational age in effort to appreciate potential impact of carnitine stores on early postnatal adaptation,
d) to compare and assess advantages and disadvantages of spectrophotometric and polarographic methods in the diagnostics of mitochondrial diseases,
e) to find out whether isolated platelets and lymphocytes from placental part of umbilical cord are usable biological material for the measurement of activities of respiratory chain complexes in neonates.

3. MATERIALS

3.1. Biological samples

3.1.1. Mitochondrial energetic metabolism in premature neonates

Isolated muscle mitochondria were used in the study. Sample of muscle tissue (m.triceps surae) was obtained after informed consent at autopsy 1-2 hours after death from 19 premature neonates (12 boys and 7 girls, 2x twins) with birth weight 790 ± 295 g (range 380-1460 g) and gestational age 25 ± 3 weeks (range 23 –35 weeks). Thirteen neonates were delivered by the Caesarean section, the other six vaginally. Seven neonates were born after premature rupture of membranes, eleven neonates due to mother´s sepsis, chorioamniitis or initiation of premature parturition with unsuccessful tocolysis, one girl was delivered due to preeclamsia in the mother. The most common clinical complications were enteral sepsis or necrotizing enterocolitis after one or two weeks of relatively good clinical condition (9 neonates), severe hypoxic-ischemic encephalopathy (1 neonate), intracranial haemorrhage (5 neonates), apoplexia (2 neonates). Anaemia, thrombocytopenia, leucopenia as a consequence of bone marrow failure and low glucose tolerance resulting in hyperlactacidaemia were found in most patients. All neonates were treated at NICU including ventilatory support, antibiotics, catecholamines and other. Most neonates died within the first month of life, the oldest infant at the age of 62 days. Fourteen patients died due to multi-organ dysfunction syndrome, two

due to cardiac failure, two due to intracranial haemorrhage and one due to meningitis with systemic inflammatory response (Honzik *et al.,* 2006).

Two control groups of children were established from 20 "disease free controls" at the age of 0.5-2 years and 26 "disease free" controls at the age of 3-18 years. These children were investigated due to clinical suspicion of neuromuscular diseases and were assigned retrospectively after no evidence of PDH deficiency and respiratory chain disorders were detected. Muscle biopsies were obtained from the m. triceps surae or m. tibialis anterior (Wenchich *et al*., 2002).

3.1.2. Mitochondrial energetic metabolism in miscarriages

Isolated muscle mitochondria were used in the study. Sample of muscle tissue (m.triceps surae) was obtained after informed consent at autopsy immediately after abortion from 18 miscarriages. Aborted fetuses were subdivided into two groups: infectious (5x) and non-infectious (13x) according to the mother's clinical status before the abortion and indication for abortion. In the group of infectious miscarriages the mean birth weight was 334 ± 241 g (range 60-700 g) and gestational age at birth 19 weeks (range 15-23 weeks of gestation) and in the group of non-infectious miscarriages the mean birth weight was 301 ± 305 g (range 30-1270 g) and gestational age at birth 19 weeks (range 15-26 weeks of gestation). All miscarriages were delivered vaginally. In the group of infectious miscarriages the cause of abortion was chorioamniitis, mother's sepsis or intrauterine infection with parvovirus B_{19} and development of non-immune hydrops fetalis. In the group of non-infectious miscarriages various reasons were indication for terminating of the pregnancy like trisomy 21, acute psychosis of the mother, anencephalus and others congenital defects. The abortion was initiated by induction of delivery using uterotonic treatment (prostaglandines), in three cases the life of the fetus was terminated by intraumbilical injection of KCl solution. The time from induction of abortion to parturition was determined (the range was very wide from few minutes to 29 hours).

3.1.3. Specific properties of heavy fraction of mitochondria from human-term placenta and glycerophosphate-dependent hydrogen peroxide production

Isolated placental mitochondria were used in the study. Placental tissue was obtained immediately after delivery from five healthy term neonates (Honzik *et al.,* 2006).

3.1.4. Carnitine concentrations in term and premature neonates

Altogether, 60 neonates were investigated: 33 healthy term neonates (16 boys, 17 girls, birth weight 3485 ± 309 g) and 27 premature neonates (12 boys, 15 girls, gestational age 24-37 week, birth weight 1855 ± 765 g, range 590-2620 g). All term neonates and 19 premature neonates were delivered vaginally, 8 premature neonates were delivered by Caesarean section. Early postnatal adaptation in all term neonates was uneventful with the Apgar score at least 7 and 9 in the first and the fifth minute, respectively. Nine mothers of term neonates and 11 mothers of premature neonates received antibiotics before the delivery due to the premature rupture of membranes (>18 hour). Five mothers were smokers during pregnancy, no one was vegetarian. All children, except three premature neonates who developed multi-organ failure, had good clinical outcome. All term neonates were breast fed and the mean milk intake of 100-120 ml/kg of body weight was achieved at the end of the first week of life. Parenteral nutrition without carnitine supplementation in combination with gradual increase in the amount of mother milk or adapted milk formula for preterm newborns was initiated in premature neonates and the mean milk intake at the end of the first week of

life was 50 ml/kg/day (range 20-100 ml/kg/day) according to individual tolerance. Small-for-gestational-age neonates and neonates with congenital malformations, perinatal asphyxia or metabolic diseases, and children of mothers with gestational diabetes mellitus or symptoms of acute infection were excluded from the study (Honzik *et al.*, 2005).

3.1.5. Polarographic and spectrophotometric studies in isolated muscle mitochondria and in permeabilized human muscle fibers

Isolated muscle mitochondria and saponin-permeabilized muscle fibers obtained from 6 patients with mitochondrial defect were used in the study. These patients (1-28 years old) had clinical symptoms of mitochondrial disorders including muscle weakness, progressive hypotony, myopathy, encephalopathy, cardiomyopathy, psychomotor retardation and lactic acidosis. In two patients, molecular analysis revealed a mutation in mtDNA. In patient 2 with clinical symptoms of the Kearns-Sayre syndrome (muscle weakness, chronic progressive external ophtalmoplegia, A-V heart block) a large scale mtDNA deletion (7.4 kB) was observed. In patient 3 with clinical symptoms of MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) a heteroplasmic mtDNA mutation 3243A>G was found (the level of heteroplasmy was 68 %). In other patients molecular analysis revealed neither mtDNA mutations 3243A>G, 8344A>G and 8993T>G nor any large-scale mtDNA deletion or mtDNA depletion. Muscle biopsies (cca 120-150 mg of muscle tissue) were obtained from the musculus tibialis anterior after informed consent of the patients or from their parents. The control group consisted of 26 patients (3-18 years old) who were recommended for muscle biopsy with the clinical suspicion of mitochondrial neuromuscular diseases, which were not confirmed (Wenchich *et al.*, 2003).

3.1.6. Mitochondrial energetic metabolism in blood cells

Lymphocytes were isolated from placental part of umbilical cord obtained after delivery of placenta in 38 healthy term neonates (20 boys, 18 girls) and 17 premature neonates (7 boys and 10 girls) with birth weight 1652 ± 728 g (range 590-2620 g) and gestational age 23-37 week. All term neonates and 10 premature neonates were delivered vaginally, 7 premature neonates were delivered by Caesarean section. In all term neonates early postnatal adaptation was uneventful with the Apgar score at least 7 and 9 in the first and the fifth minute, respectively. Nine neonates were born after premature rupture of membranes, eight neonates due to mother´s sepsis, chorioamniitis or initiation of premature parturition with unsuccessful tocolysis. Control group of children were established from 37 healthy children at the age of 0.5-2 years.

Platelets were isolated from peripheral blood in 161 children, adolescent and young adults (70 boys/men and 91 girls/women). According to the age four subgroups were selected: 32 infants and toddlers (0.5-2 years of age), 50 small children (3-9 years of age), 39 adolescent (10-19 years of age) and 40 young adults (20-25 years of age). Patients with clinical suspicion of mitochondrial disorders were excluded from the study (Böhm *et al.,* 2003).

3.2. Mitochondria and muscle fibers isolation

3.2.1. Mitochondria were isolated according to (Makinen and Lee, 1968) but without use of protease. Tissue samples were homogenized at 4˚C in a KCl medium. The homogenate was centrifuged for 10 min at 4˚C and 600 *g*, the supernatant was filtered through a 100 μm nylon screen and mitochondria were sedimented by centrifugation for 10 min at 4˚C and 10 000 *g*. The mitochondrial pellet was washed by centrifugation and resuspended to a final protein concentration of 20-25 mg/ml.

3.2.2. Muscle fibers were separated mechanically according to Kunz *et al*., (1993) in a medium containing 10 mM Ca-EGTA (0.1 μM free Ca^{2+}), 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1. Bundles of fibers between 15 and 20 mg wet weight were placed into 2 ml of an ice-cold medium. Thereafter, 50 μg/ml saponin (Sigma S-2149) was added and the suspension was gently mixed for 30 min at 4˚C. The fiber bundles were washed to remove saponin and stored on ice before being used for respiration measurements.
3.2.3. Mitochondria from human-term placenta were isolated according to (Martinez *et al.*, 1997). For better separation of heavy and light mitochondria the pH of the resolving medium was increased to 7.8 (Olivera *et al.*, 1975). Placental samples were dissected and washed several times in washing medium. Washed placenta was homogenized at 0˚C in the isolation medium using a glass-Teflon homogenizer. A 15-20% homogenate was centrifuged at 4˚C for 10 min at 750 *g*. Supernatant was filtered through gauze and centrifuged at 4˚C for 10 min at 10 800 *g*. The mitochondrial pellet was suspended in resolving medium and centrifuged for 10 min at 12 000 *g*. The pellet obtained consists of two phases, heavy and light fraction of mitochondria. Both phases were carefully separated, suspended in the isolation medium and centrifuged at 4˚C for 10 min at 10 800 *g*. After the last washing, heavy and light fractions were suspended in isolation medium and used for analysis.

3.3. Blood cells isolation

Peripheral mononuclear cells from EDTA-treated umbilical cord blood were separated on a Ficoll gradient with Lymphopack tubes (Sigma). Platelets were isolated form 9 ml of Na-citrate peripheral blood by differential centrifugation (Fox *et al*., 1992).

4. METHODS

4.1. Metabolities

4.1.1. Lactate and pyruvate were measured using monotests (Boehringer).

4.1.2. Free carnitine (FC), acylcarnitines (AC) and total carnitine (TC) were analysed in collaboration with Petr Chrastina from Institute of Inheritied Metabolic Disorders by two different methods - electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) and radioenzymatic technique (RE). All samples from dried blood spots were investigated using API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX) with TurboIonSpray (TIS) interface in combination with a PE 200 Autosampler and a PE series 200 microgradient system was used ((Hardy *et al.,* 2001). Carnitine in plasma was assayed by a radioenzymatic technique on Tri-Carb Liquid Scintillation Analyzer Models 2500TR (Cambera Packard) (McGarry *et al.,* 1976).

4.2. Protein determination

Mitochondrial proteins were determined according to (Lowry *et al*., 1951) using bovine serum albumin as a standard.

4.3. Spectrophotometric determination of mitochondrial enzyme activity

The activities of mitochondrial enzymes, NADH-coenzyme Q_{10} oxidoreductase (NQR, complex I), NADH-cytochrome *c* oxidoreductase (NCCR, complex I+III), succinate-coenzyme Q_{10} oxidoreductase (SQR, complex II), succinate-cytochrome *c* oxidoreductase (SCCR, complex II+III), coenzyme Q_{10}-cytochrome c oxidoreductase (QCCR, complex III), cytochrome c oxidace (COX, complex IV), glycerophosphate-cytochrome *c* oxidoreductase (GCCR) and cytrate synthese (CS) were measured spectrophotometrically in isolated muscle and placental mitochodria and in isolated lymphocytes and platelets (Rustin *et al*., 1994). The activity of the PDH complex was estimated as the production of $^{14}CO_2$ produced by decarboxylation of [1-^{14}C]-pyruvate (Constantin-Teodosiu *et al.*, 1991).

4.4. Electrophoretic and Western blot analyses

Blue-native electrophoresis was used for separation of mitochondrial proteins in the first dimension and SDS electrophoresis in the second dimension (Klement *et al.*, 1995). Subunits of PDH were detected by Western blot, using chicken polyclonal antibodies against the PDH holoenzyme. Immune complexes were detected with the aid of secondary peroxidase-conjugated antibody and enhanced chemiluminescence (Amersham, Little Chalfont, UK)

4.5. Polarographic determination of oxygen consumption

Oxygen consumption was measured at 30˚C using OROBOROS Oxygraph (Anton Paar, Innsbruck, Austria). The respiration of isolated muscle mitochondria was measured in the medium containing 0,5 mM EDTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 200 mM sucrose, BSA 1 g/l, pH 7.1. For saponin-skinned muscle fibers, a medium containing 0.5 mM Na_2EDTA, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 110 mM mannitol, 60 mM Tris-HCl, pH 7.4 was used. Freshly isolated or frozen-thawed human-term placental mitochondria were added to 2 ml of medium containing 100 mM KCl, 10 mM Tris-HCl, 4 mM K-phosphate, 3 mM $MgCl_2$ and 1 mM EDTA, pH 7.4. Polarographic measurements were performed as multiple substrate-inhibitor analyses (Kunz *et al*., 1993, Sperl *et al*., 1994) in the presence of 1 mM ADP. Oxygen consumption was expressed as pmol oxygen/s/mg protein. Oxygraphic curves are presented as the negative first derivation of oxygen concentration changes.

*4.6. Immunoblot analyses**

The samples of human-term placenta, hamster brown fat, rat liver and muscle mitochondria for SDS-PAGE were boiled for 3 min in a sample-lysis buffer. SDS-Tricine electrophoresis (Schagger *et al.*, 1987) was performed on 10% polyacrylaminde slab gels (Mini protean, BioRad) using the same protein aliquots of SDS-solubilized mitochondria (5-10 μg/slot). Proteins from the gel were blotted onto nitrocellulose membrane (Hybond ^{TM} C EXTRA, Amersham Bioscience) by semi-dry electrotransfer at 0.3-0.7 mA/cm^2 for 1 hour.

For immunodetection of respiratory chain enzymes, the membranes were blocked with PBS (0.15 M NaCl, 0.02 M Na_2HPO_4, pH 7.4) plus 0.2% Tween 20 (PBST) and incubated for 2.5 h with the primary monoclonal antibodies against subunits of complexes I, II, III, IV and V, respectively, diluted in PBST containing 2% bovine serum albumin. For detection of mGPDH, the membranes were blocked over night with 3% defatted milk and 0.3% Triton X-100 in 0.15 M NaCl and 0.02 M Na_2HPO_4 and incubated for 3 h with rabbit polyclonal anti-

mGPDH antibody (1:5000 dilution) raised against C-terminal peptide LDRRVPIPVDSCGG of mouse enzyme according to (Ueda *et al.*, 1998). Then the membranes were incubated for 1.5 h using either goat anti-mouse IgG (1:1000, A8924 Sigma) or goat anti-rabbit IgG (1:1000, F0382 Sigma) secondary antibodies conjugated with horseradish peroxidase. The chimiluminescent reaction using ECL kit (Amersham Biosciences) was detected on a LAS 1000 (Fujifilm) and signal was quantified using Aida 2.11 Image Analyser software (Raytest).

*4.7. Fluorometric detection of hydrogen peroxide production **

For determination of hydrogen peroxide production fluorescent probe dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) was used. Mitochondria were incubated in 1 ml of KCl-medium containing 0.05 mg of mitochondrial protein, 1 μM H_2DCFDA, 1 μM antimycin A and glycerophosphate, succinat or NADH as indicated. Fluorescence was determined at excitation wavelength of 425 nm and emission wavelength of 520 nm using Wallac Victor^2 fluorescence plate-reader.

*The analyses were performed at Dept.of Bioenergetics, Institute of Physiology, Academy of Science, Czech Republic

4.8. Statistical Analysis

Statistical analysis used in the study characterizing carnitine profile in premature neonates: Student's test for unpaired samples was used to test the differences in carnitine level in cord plasma and the whole cord blood. Simple linear regression was used to test for the correlation between carnitine level and gestational age, birth weight or blood count. Differences were regarded as significant at a $p<0.05$. T-test for paired samples was used to compare the changes in carnitine level in cord blood and Guthrie cards.

Statistical analysis used in the study characterizing activities of RC complexes in premature neonates: Program Statgraphics Plus version 6.0 was used for statistical analysis. The results of enzymatic investigations are expressed as mean ± SD. Statistical significance for comparison between groups was assessed using the nonparametric Mann-Whitney U test and a *p*-value of <0.05 was considered significant. A simple linear regression was used to test for the correlation between enzyme activities and weight or gestational age.

5. RESULTS AND DISCUSSION

5.1. Mitochondrial energetic metabolism in neonates

Age dependent differences in the activities of respiratory chain complexes and PDH were observed during childhood. In the premature neonates, the specific activities of respiratory chain complexes III, IV, PDH and CS in isolated muscle mitochondria were significantly lower in comparison with older children (Figure 1). On the contrary, the activity of complex I was higher in premature neonates in comparison with older children (Figure 1) (Honzik *et al.,* 2006).

The PDH activity was significantly lower (3.9 ± 2.2 nmol/min/mg protein) in the group of 10 premature neonates with severe hyperlactacidemia (>5.9 mmol/l, controls < 2.3 mmol/l) in comparison with 9 premature neonates (6.1 ± 2.4 nmol/min/mg protein, $p<0.05$) with normal or only mildly increased lactate.

Blue-native electrophoresis of respiratory chain complexes in isolated muscle mitochondria in premature neonates revealed decreased protein amount of respiratory chain

complexes I, III, IV and V in all 15 analysed premature neonates in comparison with controls. Data of four very premature neonates are shown in Figure 2.

Using Western blot, a lower amount of E1-alfa, E1-beta, protein X and E2 subunits of pyruvate dehydrogenase (20-50 % of control value) were found in all 9 analysed premature neonates in comparison with older controls. Data of three very premature neonates are shown in Figure 3.

The low activities of RC complexes III and IV and pyruvate dehydrogenase in muscle mitochondria observed in our study in premature neonates seem to correspond to the low protein amount of these mitochondrial enzymes. We are aware of the fact that the age of death in our group of premature neonates varied. To what extent the secondary factors such as sepsis may have any influence on our results can be discussed, but animal studies and scarce studies in human neonates (Cuezva *et al.,* 1992, Sperl *et al.,* 1992, Valcarce *et al.,* 1994, Smeitink *et al.,* 1992) provide explicit evidence of lower mitochondrial protein synthesis in premature individuals. An increased level of lactate is often found in neonates as a result of hypoxia, low blood perfusion, hepatic or renal failure, high glucose intake and also in children with various inherited metabolic disorders (Hutchesson *et al.,* 1997, Stern, 1994). The low activity of PDH in premature or critically ill neonates may not only negatively influence the ATP production but it may also be the cause of low glucose tolerance and lactic acidosis (Vary, 1996). It is in agreement with our results of premature neonates with severe hyperlactacidemia where we found lower activity of PDH in comparison with premature neonates in which lactic acidosis were not so accentuated.

Most diseases in premature neonates are secondary to infection and immaturity of various organ systems. The results of our study document the age dependent differences in activities of PDH and respiratory chain complexes in early childhood. Lower functional capacity of mitochondrial energy providing system in critically ill premature neonates may be explained by combination of various factors including the delay in maturation of PDH and respiratory chain complexes in very premature neonates and increased degradation of mitochondrial proteins in connection with sepsis, tissue hypoperfusion or hypoxemia (Honzik *et al.,* 2006).

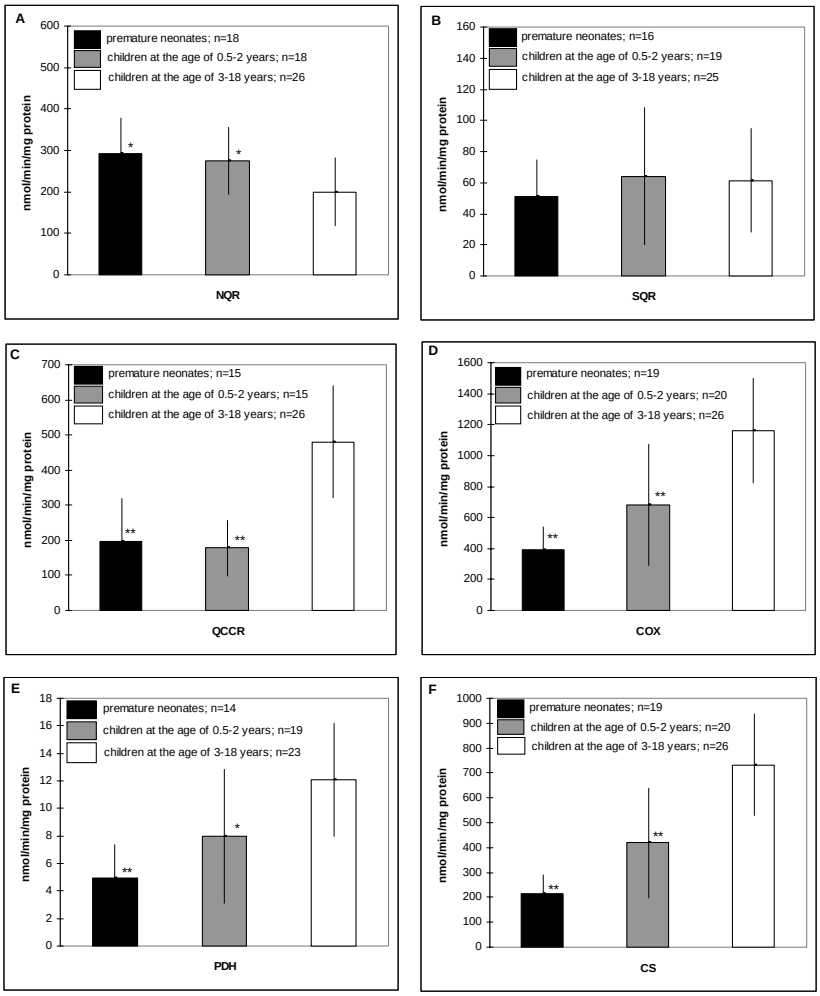

Figure 1. *Activities of respiratory chain complexes and pyruvate dehydrogenase in isolated muscle mitochondria in 19 premature neonates in comparison with older children at the age between 0.5 and 2 years or 3 and 18 years. NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), SQR: succinate-coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome c oxidoreductase (complex III), COX: cytochrome c oxidase (complex IV), PDH: pyruvate dehydrogenase , CS: citrate synthase. The differences between age groups are marked with asterisk and the level of significance: *p < 0.01, **p < 0.001.*

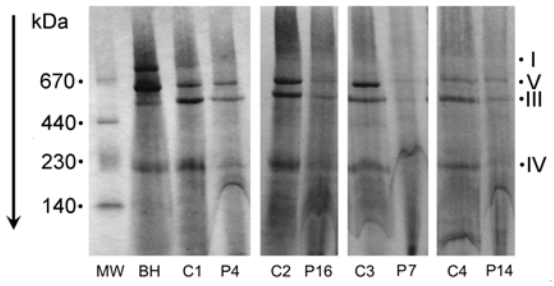

Figure 2. *Blue-Native electrophoresis of respiratory chain complexes in isolated muscle mitochondria in four very premature neonates in comparison with controls. Protein aliquots of lauryl-maltoside-solubilised isolated mitochondria (15 µg) from bovine heart (BH), four very premature neonates (P4, P7, P14, P16) and four adult controls (C1-4) were analysed on a 5-10 % polyacrylamide gradient gel and stained with Coomassie Brilliant Blue R. The migration of molecular mass standards (MW) and the position of respiratory chain complexes I, III, IV and V are indicated.*

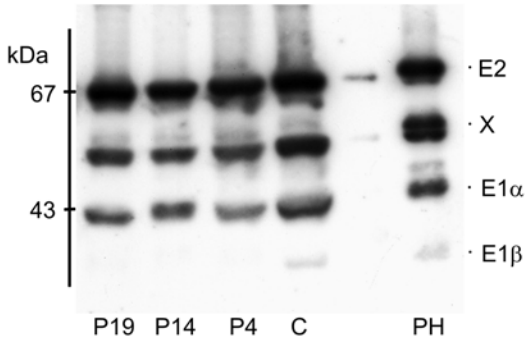

Figure 3. *Western blot analysis of the pyruvate dehydrogenase complex in isolated muscle mitochondria. 15 μg protein aliquots of isolated mitochondria from three very premature neonates (P4, P14, P19), adult control (C) and 4.5 μg protein of isolated pyruvate dehydrogenase from porcine heart (PH) were analysed by SDS PAGE, transferred to nitrocellulose membrane and probed with anti-PDH antibodies. Positions of PDH subunits E2, X, E1α and E1β are indicated. PDH - pyruvate dehydrogenase complex, E1 - pyruvate decarboxylase, E2 - dihydrolipoamide transacetylase, X - subunit X (E3 binding protein).*

5.2. Mitochondrial energetic metabolism in miscarriages

The specific activities of PDH and respiratory chain complexes in the isolated mitochondria from the muscle tissue of 13 non-infectious miscarriages (gestational age in the range from 15th to 26th weeks) and 5 infectious miscarriages (gestational age in the range from 15th to 23th weeks) in comparison with 19 premature neonates (gestational age in the range from 23rd to 35th weeks) and the controls aged 0.5-2 years are shown in Table 4 A, B and Figure 4 A, B. In the 19 premature neonates, 5 infectious miscarriages and 13 non-infectious miscarriages the specific activity of respiratory chain complex IV and CS, and the specific activities of respiratory chain complexes I, II and III in 13 non-infectious miscarriages, and the specific activity of PDH in premature neonates were significantly lower in comparison with the control group of children at the age of 0.5-2 years.

In the group of 13 non-infectious miscarriages in comparison with premature neonates significantly lower specific activities of respiratory chain complex I, II, III, IV and CS were found. In contrast, specific activity of PDH was significantly higher in non-infectious miscarriages in comparison with the group of premature neonates (Table 4 A, B).

In effort to evaluated the potential influence of infectious complications on activities of respiratory chain complexes we compared group of infectious (5x) and non-infectious miscarriages (13x) and septic (9x) and non-septic (10x) premature neonates. The specific activities of respiratory chain complexes I (320.7 ± 132.7, 222.9 ± 76.5 nmol/min/mg protein, $p<0.05$) and complex II (70.84 ± 84.3, 29.6 ± 12 nmol/min/mg protein, $p<0.05$) in isolated muscle mitochondria were significantly higher in the infectious group of miscarriages in comparison with the others. No significant differences were found between individual activities of all analysed respiratory chain complexes in septic and non-septic premature neonates (data not shown).

A simple linear regression analysis revealed significant positive correlation between specific activity of complex IV and CS and gestational age and specific activity of complex IV and birth weight in premature neonates plus non-infectious miscarriages. (Figure 5 A, B, C, D).

Blue-native electrophoresis of respiratory chain complexes in isolated muscle mitochondria in premature neonates and miscarriages revealed decreased protein amount of respiratory chain complexes I, III, IV and V in all 15 analysed premature neonates and 10 analysed miscarriages in comparison with controls. Data of five patients (one premature neonate, two infectious and two non-infectious miscarriages) are shown in Figure 6.

Using Western blot, a lower amount of E1-alfa, E1-beta, protein X and E2 subunits of pyruvate dehydrogenase (20-50 % of control value) were found in all 9 analysed premature neonates and 10 analysed miscarriages in comparison with older controls. Data of three non-infectious and one infectious miscarriage and three premature neonates are shown in Figure 7. Both methods western blot and blue native electrophoresis, demonstrated positive correlation between protein synthesis and gestational age.

While low capacity of mitochondrial energy providing system in critically ill premature neonates may be partly explained by increased degradation of mitochondrial proteins in connection with sepsis, hypoxia or tissue hypoperfusion, our study on aborted fetuses clearly demonstrate the maturation processes of energy provision during fetal period suggesting the impact of immaturity on mitochondrial functions.

Table 4. Comparison of specific activities of respiratory chain complexes and pyruvate dehydrogenase and their ratio to succinate-coenzyme Q_{10} oxidoreductase (SQR) in 19 premature neonates, 13 non-infectious miscarriages and 5 infectious miscarriages with 20 controls aged 0.5-2 years (A) and their statistical parameters (B).

A.

Group	A		B		C		D	
	Premature neonates		Infectious Miscarriages		Non-infectious Miscarriages		Controls	
Gestat. age (days)	178,7 ± 20,38		134 ± 24		137 ± 20		0,5-2 years	
Birth weight (grams)	790 ± 295		334 ± 241		301 ± 305			
Enzymes activities	nmol/min/protein							
	n		n		n		n	
COX	19	391,7 ± 148,3	5	235,7 ± 124,6	13	164,1 ± 71,1	20	681,8 ± 394,7
CS	19	214,9 ± 74,9	5	133,1 ± 68,2	13	90,4 ± 38	20	419 ± 221
NQR	18	292,9 ± 84,8	5	320,7 ± 132,7	13	222,9 ± 76,5	18	274 ± 80,7
SQR	16	50,9 ± 23,4	5	70,84 ± 84,3	13	29,6 ± 12	19	63,9 ± 44,2
QCCR	15	195,3 ± 122,7	5	141,1 ± 57	13	109,2 ± 24,7	14	178,3 ± 79,2
PDH	14	4,9 ± 2,5	5	5,53 ± 1,9	12	7,2 ± 4,2	19	8 ± 4,86
COX/SQR	16	10,7 ± 10,3	5	6,3 ± 4,9	13	6,3 ± 3,2	19	13,2 ± 8,4
CS/SQR	16	5 ± 2,8	5	3,6 ± 2,2	13	3,4 ± 1,7	19	8,4 ± 4,8
NQR/SQR	16	7,9 ± 6,2	5	9,1 ± 5,8	13	8 ± 2,5	18	6 ± 3,2
QCCR/SQR	13	4,1 ± 2,8	5	3,7 ± 2,1	13	4 ± 1,3	14	4 ± 2,1
PDH/SQR	12	0,15 ± 0,1	5	0,19 ± 0,11	12	0,24 ± 0,07	18	0,16 ± 0,1

NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), SQR: succinate-coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome *c* oxidoreductase (complex III), COX: cytochrome *c* oxidase (complex IV), PDH: pyruvate dehydrogenase, CS: citrate synthase

B.

Group	A:B	A:C	A:D	B:C	B:D	C:D
Complexes	*p*-value					
COX	< 0,025	< 0,001	< 0,005	NS	< 0,025	< 0,001
CS	< 0,025	< 0,001	< 0,005	NS	< 0,005	< 0,001
NQR	NS	< 0,025	NS	< 0,05	NS	< 0,05
SQR	NS	< 0,005	NS	< 0,05	NS	< 0,01
QCCR	NS	< 0,01	NS	NS	NS	< 0,005
PDH	NS	< 0,05	< 0,025	NS	NS	NS
COX/SQR	NS	NS	NS	NS	< 0,05	< 0,005
CS/SQR	NS	< 0,05	< 0,01	NS	< 0,025	< 0,001
NQR/SQR	NS	NS	NS	NS	NS	< 0,05
QCCR/SQR	NS	NS	NS	NS	NS	NS
PDH/SQR	NS	< 0,01	NS	NS	NS	< 0,025

NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), SQR: succinate-coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome *c* oxidoreductase (complex III), COX: cytochrome *c* oxidase (complex IV), PDH: pyruvate dehydrogenase

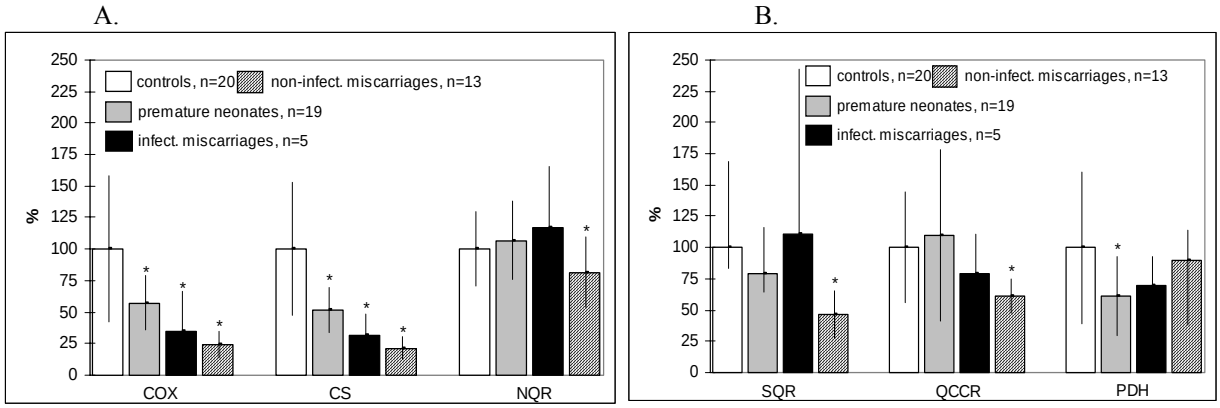

Figure 4. *Comparison of relative activities of respiratory chain complexes and pyruvate dehydrogenase in 19 premature neonates, 13 non-infectious miscarriages and 5 infectious miscarriages with 20 controls aged 0,5-2 years. The activities in the premature neonates and miscarriages are expressed in percents of the mean enzyme activities of the control group of children aged 0,5-2 years. The respective specific activities (nmol/min/mg protein) in children 0,5-2 years are: COX 681,8 ± 394,7, CS 419 ± 221, NQR 274 ± 80,7, SQR 63,9 ± 44,2, QCCR 178,3 ± 79,2, NCCR 98,8 ± 57,3, SCCR 127,9 ± 77,9, PDH 8 ± 4,86.* * $p < 0.05$

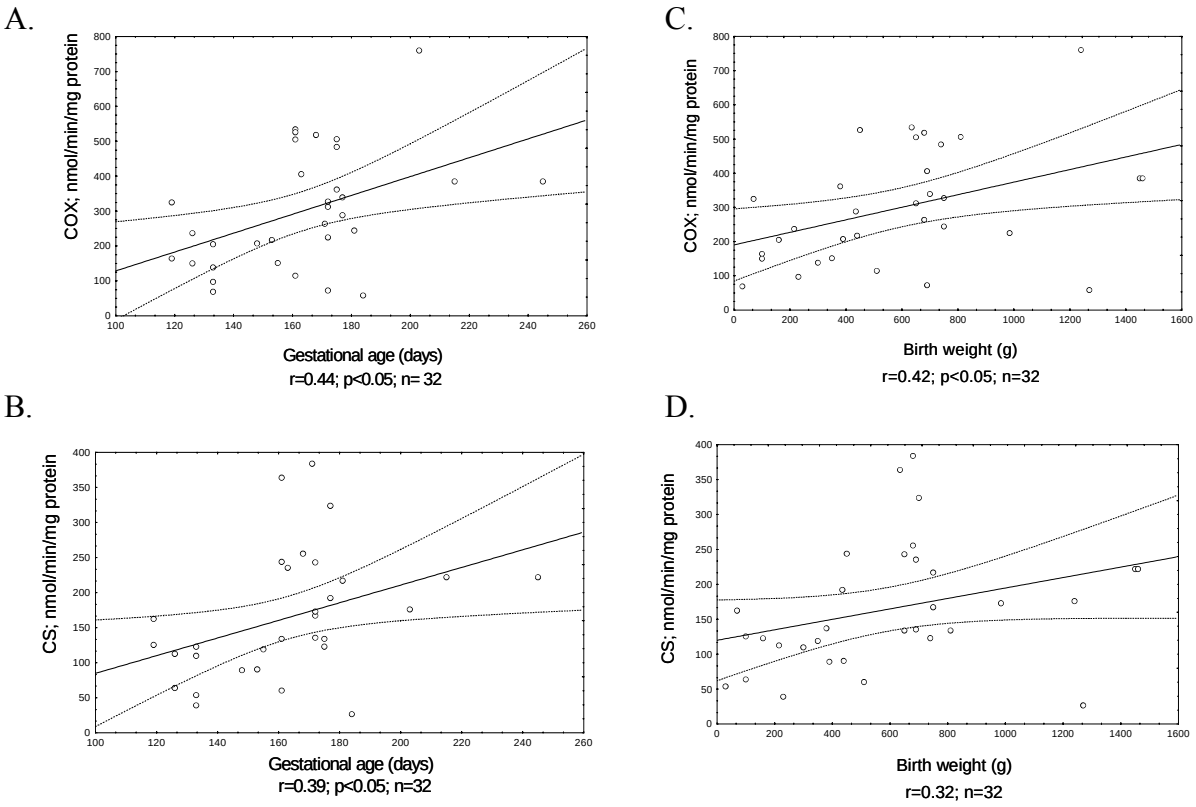

Figure 5. *The correlation between specific activity of cytochrome c oxidase (complex IV) (A, C) or citrate synthase (CS) (B, D) in premature neonates and non-infectious miscarriages and gestational age or birth weight.*

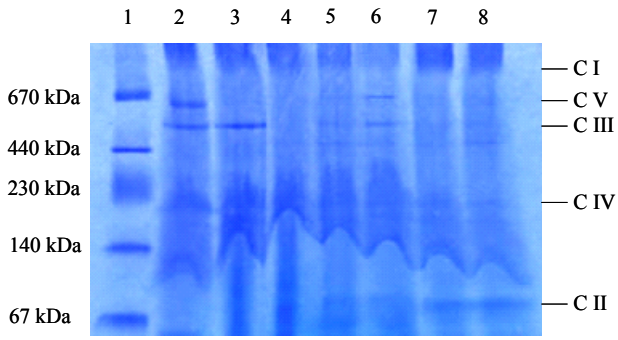

Figure 6. *Electrophoretic analysis of the respiratory chain complexes by Blue native electrophoresis. 20-ug protein aliquots of lauryl-maltoside-solubilized isolated mitochondria from adult control (lane 2), disease free patient 1 year old (lane 3), premature neonates (24+4 wk) (lane 4), non-infectious miscarriages (18 and 26+2 wk) (lanes 5, 6), infectious miscarriages (19 and 15+5 wk) (lanes 7, 8) and protein markers indicated molecular weight (lane 1) were analysed on a 5-10% poly-acrylamide gradient gel and stained with Coomassie Brilliant Blue R. The position of the respiratorychain complexes I-V is indicated on the right. The migration of the molecular weight standarts on the left.*

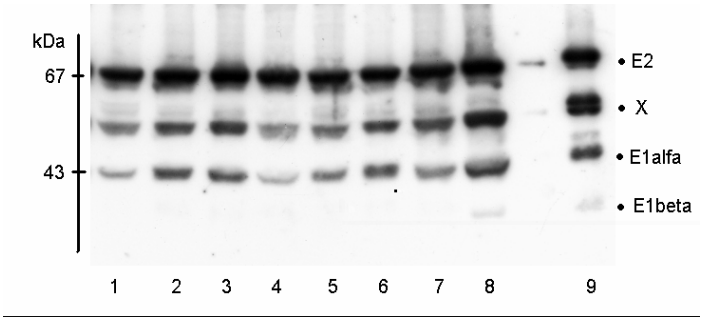

Figure 7. *Western blot analysis of the PDH complex in isolated muscle mitochondria. 15 ug protein aliquot of isolated mitochondria from control (line 8), infectious miscarriage (23 wk of gestation) (line 4), non-infectious miscarriages (19, 19, 18 wk of gestation) (line 1, 2 and 5), premature neonates (24+4, 24+4 and 25 wk of gestation) (line 3, 6 and 7) and 4,5 ug protein of isolated porcine heart (line 9)were analysed by SDS PAGE, transferred to mitrocellulose membrane and probed with anti PDH antibody. Positions of PDH subunits E2, X and E1 alfa and beta are indicated. PDH = pyruvate dehydrogenase complex, E1 = pyruvate decarboxylase, E2 = dihydrolipoamide transacetylase, X = subunit X.*

5.3. Specific properties of heavy fraction of mitochondria from human-term placenta and glycerophosphate-dependent hydrogen peroxide production

5.3.1. Activity and quantity of various mitochondrial enzymes in placental mitochondria

In published data, there are significant discrepancies related to oxidation of glycerophosphate, succinate and NADH dependent substrates. Similarly as in brown fat mitochondria (Houstek *et al.,* 1975), we found the rate of glycerophosphate-cytochrome *c* oxidoreductase (GCCR) activity in placenta is quite comparable to that of SCCR (Table 5). When oxygraphic determination of succinate-or glycerophosphate-dependent respiration was tested, we found similar rates of respiration with both substrates (Table 6). We thus confirmed

previous findings demonstrating high activity of mGPDH in human placental mitochondria (Olivera *et al.,* 1975, Gellerich *et al.,* 1994, Swierczynski *et al.,* 1976a, b). Determination of NCCR showed that the rotenone-insensitive NCCR activity was almost four-fold higher than GCCR or SCCR (Table 5). However, the rotenone-sensitive fraction was quite comparable to the activity of GCCR or SCCR. High rotenone-insensitive activity is evidently connected with a high microsomal contamination as observed also in previous studies (Gellerich *et al.,* 1994, Gasnier *et al.,* 1993) or with high activity of NCCR of outer mitochondrial membrane, which is also rotenone insensitive (Sottocasa *et al.,* 1967). Also using oxygraphic studies we found that the rate of glutamate and malate or pyruvate and malate respiration is only about 50% of that of succinate- or glycerophosphate-dependent respiration (Table 6). This discrepancy between spectrophotometric and oxygraphic measurements could indicate some limitations in transport of NADH-dependent substrates to placental mitochondria or modified function of NADH oxidase. To further investigate this hypothesis we measured rotenone-sensitive NADH oxidation by oxygraphy. In frozen-thawed placental mitochondria we found the rate of NADH oxidation (Table 7) to be three to four times lower than the rate of glycerophosphate or succinate oxidation. These results suggest lower content or down-regulated activity of complex I in placental mitochondria. We therefore determined specific content of individual respiratory chain complexes by Western blot analysis using subunit-specific monoclonal antibodies against RC complexes. As demonstrated in Figure 8A, the relative proportion between individual complexes was quite similar in human placenta, mouse liver and mouse muscle mitochondria. Apparently, the content of CI in placenta was quite comparable with that in other tissues. Furthermore, we used the same approach to quantify the content of mGPDH. As apparent from Figure 8B, placental mitochondria contained about two-fold higher amount of mGPDH than liver mitochondria which corresponded to about 38% of the mGPDH content in brown adipose tissue mitochondria.

5.3.2. Glycerophosphate-dependent hydrogen peroxide production in placental mitochondria

In the previous studies (Drahota *et al.,* 2002, Drahota *et al.,* 2003) was found that mGPDH represents a new site of ROS production in the mitochondrial respiratory chain. Using fluorometric detection of hydrogen peroxide generation by H2DCFDA we could demonstrate a high rate of glycerophosphate-dependent hydrogen peroxide production also in placental mitochondria. Under the same experimental conditions, succinate- or NADH-dependent hydrogen peroxide production was several times less intensive (Figure 9). Similarly as in brown fat (Drahota *et al.,* 2002) and liver mitochondria (Jesina *et al.,* 2004), also in placental mitochondria we found glycerophosphate-dependent hydrogen peroxide production that was about sevenfold increased by one-electron acceptor, potassium ferricyanide (Figure 10). As shown in Figure 10, when the added ferricyanide was reduced by mGPDH, the rate of hydrogen peroxide production returned again to the original values. This activation of oxygen uptake could be repeated several times by addition of another portion of oxidized ferricyanide (Figure 10). Contrary to glycerophosphate, succinate was not able to support hydrogen peroxide generation under the same experimental conditions (Figure 11). Antimycin A was used in that experiment as the respiratory chain inhibitor and we obtained the same results as in previous experiments with KCN (Figure 10). Fifty percent inhibition of mGPDH activity by 50 mM mercaptodicarbanonaborate (Drahota *et al.,* 1995) led to decline in hydrogen peroxide production rate (Figure 12), however, the total amount of hydrogen peroxide produced with respect to added ferricyanide remained unchanged. The inhibition of mGPDH activity by mercaptodicarbanonaborate can be completely eliminated by bovine serum albumin (Drahota *et al.,* 1995). Similarly, addition of serum albumin after

mercaptodicarbanonaborate inhibition completely recovered the ferricyanide-induced, glycerophosphate-dependent hydrogen peroxide production (Figure 12), which confirms direct involvement of mGPDH in this reaction. Cytochrome c is known as a potent endogenous scavenger of electrons (Pereverzev *et al.,* 2003). Figure 12 demonstrates that increasing concentration of cytochrome c strongly depressed glycerophosphate-dependent hydrogen peroxide production.

5.3.3. Concluding remarks

In this work we focused on detailed characterization of mitochondria isolated from human-term placenta, in order to clarify discrepancies in published data on activities of various mitochondrial enzymes and their proportions. Activities of rotenone-sensitive NCCR, SCCR and GCCR were quite similar, however, when measured as oxygen uptake, we found in accordance with others e.g. (Gellerich *et al.,* 1994) a lower rate of oxygen uptake with NADH-dependent substrates than with succinate or glycerophosphate. Using specific antibodies we found that the amount of respiratory chain complex I is not reduced in placental mitochondria and the proportions among RC complexes I, II, III, IV and complex V are similar to those in other mammalian tissues. From our experiments and from other published data (e.g. (Gellerich *et al.,* 1994) we may conclude that mitochondria in placenta are capable of regular ATP production by coupled oxidative phosphorylation and have normal content of respiratory chain enzymes. It appears that the activity of NADH oxidase is somehow down-regulated in placental mitochondria, because both the oxidation of NADH-dependent substrates in intact mitochondria and oxidation of NADH in frozen-thawed and sonicated mitochondria is lower than that of glycerophosphate and succinate.

Very specific feature of placental mitochondria is unusually high activity of mGPDH. High activity of this enzyme enables function of the glycerophosphate shuttle (Swierczynski *et al.,* 1976) that is a very useful regulatory device which can reoxidize cytosolic NADH and maintain the high rate of glycolysis without production of lactic acid. In most mammalian tissues the expression of mGPDH is highly depressed, placental cells thus could represent a useful model system for elucidation of mechanisms controlling mGPDH biogenesis in human tissues.

Recently was found that mGPDH beside its positive role in activation of glycolysis, represents also a potential risk for mammalian cells as a generator of ROS (Drahota *et al.,* 2002, 2003). Data presented in this work show that a significant glycerophosphate-dependent hydrogen peroxide production can be detected also in placental mitochondria. Glycerophosphate-dependent hydrogen peroxide generation could participate in disturbances of placental development as well as in placental pathologic processes connected with oxidative stress (Bloxam *et al.,* 1987, Poston *et al.,* 2004).

Table 5. NADH-dependent and flavoprotein-dependent enzyme activities of human placental mitochondria

Enzyme activity	nmole/min/mg protein	% activity
Glycerophosphate: cytochrome *c* oxidoreductase	107.8 ± 1.23	100
Succinate:cytochrome *c* oxidoreductase	108.8 ± 2.92	101
NADH:cytochrome *c* oxidoreductase		
Rotenone-sensitive activity	93.8 ± 0.47	87
Rotenone-insensitive activity	395.7 ± 8.96	367

The enzyme activities were measured as described in Material and Methods. The data represent means ± SEM from analysis of four different mitochondrial preparations from human-term placenta.

Table 6. Oxygen uptake of human-term placental mitochondria with different substrates

Additions	Oxygen uptake (pmol oxygen/s/mg protein)
GP	109.4 ± 9.2
GP+cyto	169.6 ± 5.8
GP+cyto+ADP	292.3 ± 6.1
GP+cyto+ADP+FCCP	310.5 ± 19.6
GP+cyto+ADP+FCCP+KCN	22.3 ± 5.2
(RCI = 1.72±0.13)	
SUC	110.4 ± 7.3
SUC+cyto	140.7 ± 17.5
SUC+cyto+ADP	260.0 ± 21.0
SUC+cyto+ADP+FCCP	307.0 ± 23.0
SUC+cyto+ADP+FCCP+KCN	15.9 ± 5.2
(RCI = 1.84±0.15)	
GLU+MAL+cyto	61.2 ± 10.4
GLU+MAL+cyto+ADP	152.1 ± 17.0
GLU+MAL+cyto+ADP+ROT	24.4 ± 6.1
(RCI = 2.49±0.13)	
PYR+MAL+cyto	66.1 ± 7.0
PYR+MAL+cyto+ADP	130.1 ± 17.0
PYR+MAL+cyto+ADP+ROT	19.6 ± 3.5
(RCI = 1.97±0.23)	
ASC+TMPD+cyto	725.5 ± 45.6
ASC+TMPD+cyto+KCN	2.3 ± 3.2

Freshly isolated human-term placental mitochondria were incubated in 100 mM KCl, 10 mM Tris-HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.6 mg /ml bovine serum albumin fatty acid free, pH 7.3 at 30^o C . Where indicated 10 mM glycerophosphate (GP), 10 mM succinate (SUC) 10 mM glutamate (GLU), 3 mM malate (MAL), 20 μM cytochrome *c*, 1 mM ADP, 3 μM rotenone (ROT), 5 mM ascorbate (ASC), 1 mM tetramethyl-p-phenylendiamine (TMPD) were added. Protein was 0.5 mg/ml. RCI indicates respiratory control index for particular substrate. Measurements with glycerophosphate and succinate were repeated five times and measurements with glutamate, malate and ascorbate were repeated three times with different mitochondrial preparations from human-term placentas. Data indicate average and ±SEM.

Table 7. NADH, glycerophosphate and succinate oxidation by frozen-thawed placental mitochondria

Additions	Oxygen uptake (pmol oxygen/s/mg protein)
NADH	85.4
NADH+ROT	32.4
NADH+ROT+cyto	270.7
NADH+ROT+cyto+KCN	3.5
NADH	84.6
NADH+cyto	360.8
NADH+cyto+FCCP	340.0
NADH+cyto+FCCP+ROT	263.3
NADH+cyto+FCCP+ROT+KCN	2.5
*NADH	82.6
*NADH+cyto	208.1
*NADH+cyto+FCCP	216.0
*NADH+cyto+FCCP+ROT	163.9
*NADH+cyto+FCCP+ROT+KCN	4.7
GP	75.2
GP+cyto	425.3
GP+cyto+KCN	2.9
SUC	74.5
SUC+cyto	322.2
SUC+cyto+KCN	4.7

Where indicated (*) aliquot of frozen-thawed mitochondria was sonicated three times for 15 sec at 0° C. Incubation conditions were the same as in Table 7. NADH was 0.4 mM, glycerophoshte (GP) 10 mM, succinate (SUC) 10 mM, cytochrome *c* (cyto) 40 μM, rotenone (ROT) 3 μM. Mitochondrial protein was 0.4 mg/ml.

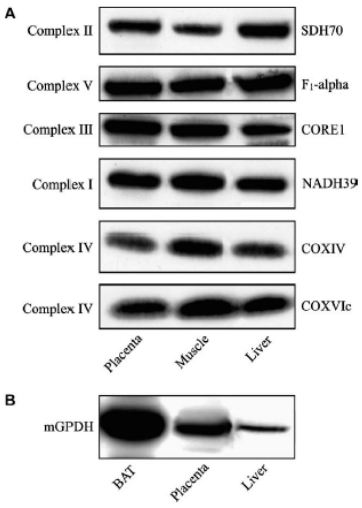

Figure 8. Immunodetection of the respiratory chain enzymes by specific antibodies. In (A), SDS-polyacrylamide gel electrophoresis and Western blot analysis was performed in isolated mitochondria from human placenta, rat muscle and rat liver using monoclonal antibodies against subunits of respiratory chain Complex I (NADH39), Complex II (SDH70), Complex III (CORE1), Complex IV (COXIV and COXVIc) and Complex V (F1-alpha). In (B), the same analysis was performed in isolated mitochondria from hamster brown adipose tissue (BAT), human placenta and rat liver mitochondria using polyclonal antibody against mGPDH.

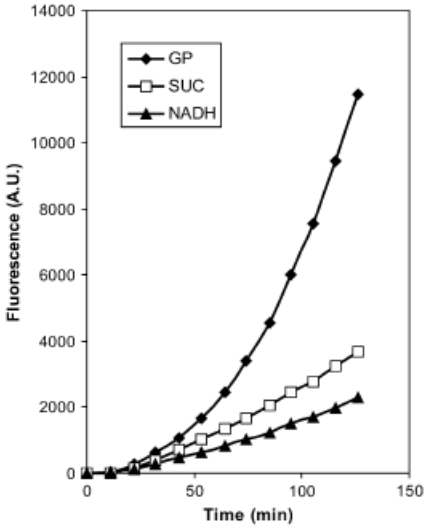

Figure 9. *Fluorometric detection of hydrogen peroxide generation by placental mitochondria. Frozen-thawed mitochondria (0.05 mg protein/ml) were incubated in 1 ml of 100 mM KCl, 20 mM TriseHCl, 4 mM Kphosphate, 3 mM MgCl2, 1 mM EDTA, pH 7.4 in the presence of 2 mM H2DCFDA and 2 mM antimycin A. Ten millimoles of glycerophosphate (GP), 10 mM succinate (SUC) or 0.4 mM NADH was used as a substrate.*

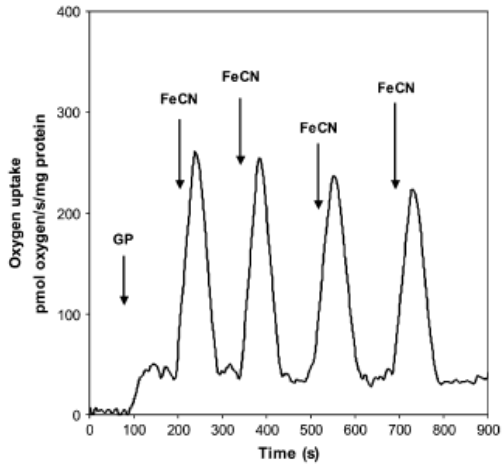

Figure 10. *Ferricyanide-activated, glycerophosphate-dependent hydrogen peroxide generation by human placental mitochondria. Freshly isolated mitochondria (1 mg protein/ml) were incubated at 30 (C in 100 mM KCl, 20 mM TriseHCl, 4 mM K-phosphate, 3 mM MgCl2, 1 mM EDTA, pH 7.4 and 1 mM KCN. Ten millimoles of glycerophosphate (GP) and 63 mM potassium ferricyanide (FeCN), were added as indicated. The experiment was repeated twice using two different placental mitochondria preparations with the same results.*

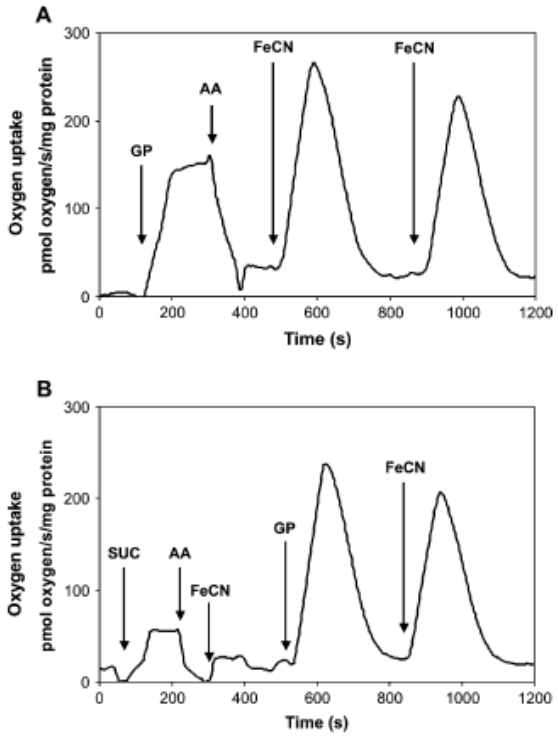

Figure 11. *Ferricyanide-induced hydrogen peroxide production in the presence of glycerophosphate (A) and succinate (B). Frozen-thawed placental mitochondria were incubated at 30 (C in 100 mM KCl, 20 mM TriseHCl, 4 mM K-phosphate, 3 mM MgCl2, 1 mM EDTA, pH 7.4. In (A), where indicated, 10 mM glycerophosphate (GP), 1 mM antimycin A (AA), and twice 63 mM potassium ferricyanide (FeCN), were added. In (B), where indicated, 10 mM succinate (SUC), 1 mM antimycin A (AA), 10 mM glycerophosphate (GP) and 63 mM potassium ferricyanide (FeCN), were added. Mitochondrial protein was 0.46 mg/ml.*

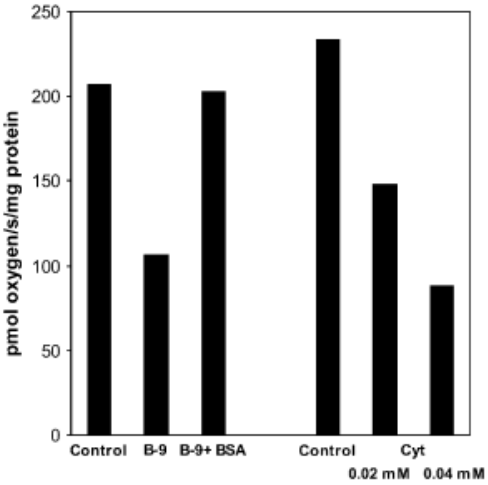

Figure 12. *Inhibition of glycerophosphate-induced hydrogen peroxide production by mercapto-dicarbanonaborate and cytochrome c. Incubation conditions were the same as in Figure 17. Mitochondrial protein was 0.5 mg/ml. KCN-insensitive oxygen uptake was induced by addition of 10 mM glycerophosphate (GP) and 63 mM potassium ferricyanide (FeCN). Glycerophosphate-dependent, FeCN-activated hydrogen peroxide production was measured in the presence of 50 mM mercaptodicarbanonaborate (B-9), or B-9 and 1 mg/ml bovine serum albumin fatty acid free (B-9CBSA). Inhibition of cytochrome c, was measured in the presence of 20 mM or 40 mM cytochrome c(Cyt). The experiment was repeated twice using two different placental mitochondria preparations with the same results.*

5.4. Carnitine concentrations in term and premature neonates

Cord plasma level of free carnitine (FC), acylcarnitines (AC) and total carnitines (TC) estimated by radioenzymatic technique was significantly higher in premature neonates in comparison with term neonates (Table 8) and negative correlation between cord plasma level of FC, AC or TC and birth weight or gestational age was found (Figure 13 C, D). On the contrary, the total blood pool of FC and TC in cord blood estimated by ESI-MS/MS method were significantly lower in premature neonates in comparison with term neonates (Table 8) and the positive correlation between cord blood level of FC and gestational age or birth weight was observed (Figure 13 A, B). No correlation was observed between red blood cell count, haematocrit or white blood cell count and the level of FC or TC in the whole cord blood (data not shown), but positive correlation was found between AC level and RBC or haematocrit (Figure 14). The TC level decreased significantly from birth to the postnatal day 4–6 in term neonates and from birth to the postnatal day 7–10 in premature neonates (Figure 15). During the same period FC level decreased in term neonates and AC level decreased in premature neonates.

The activity of respiratory chain complex I+III and CS in isolated lymphocytes obtained from umbilical cord in 17 premature neonates and 33 term neonates increased significantly with increasing free carnitine blood level and with increasing ratio FC/TC. In addition, activities of respiratory chain complexes I+III, II and CS decreased with increasing level of AC and ratio AC/FC (Figure 16).

Placental transfer is the main source of carnitine for the fetus and for the neonates during the early postnatal period. The plasma carnitine concentration in pregnant women decreases during gestation and reaches the lowest levels near the end of the gestation (Hahn *et al.,* 1977, Winter *et al.,* 1995). Similar to other studies using RE technique, we observed higher level of carnitine in cord plasma in our group of premature neonates in comparison with term neonates resulting from a higher placental carnitine transfer from the mother in earlier stages of gestation (Novak *et al.,* 1981). It was already presented using ESI-MS/MS method, that the total blood pool of carnitine in premature neonates is lower in comparison with term neonates (Meyburg *et al.,* 2001) and it futher increases during childhood (Cavedon *et al.,* 2005). We also observed higher FC and TC levels in cord blood in term neonates in comparison with premature neonates and significant correlation between FC and gestational age or birth weight. It supports the hypothesis, that increasing store of carnitine at the end of gestation is supposed for the postnatal participation of carnitine at the mitochondrial fatty acid oxidation. Lower cord blood carnitine pool in premature neonates in comparison with term neonates might indicates, that premature neonates are born with limited carnitine store (Meyburg *et al.,* 2001).

Acylcarnitines in red blood cells represent the major pool of carnitine (65-75 %) in blood (Battistella *et al.,* 1980, Cress *et al.,* 1989). Several theories tried to explain the role of carnitine in erythrocytes (Battistella *et al.,* 1980), but AC in erythrocytes may represent freely exchangeable reservoir of promptly available activated acyl-groups. We found positive correlation between AC level and red blood cell count and haematocrit. Therefore not only gestational age and fetal growth, but also changes in red blood cell count and haematocrit may influence the results of carnitine measurement if ESI-MS/MS method is used.

We compared carnitine levels in cord blood with carnitine levels in Guthrie card supposed for neonatal screening of PKU. During the first week of life, the average blood levels of TC and FC decreased in term neonates and TC and AC decreased in premature neonates. We did not found any correlation between carnitine levels in blood and the amount of milk intake during the first days of life.

In the cell, L-carnitine is involved in a variety of metabolic processes including the mitochondrial energetic metabolism and energy production. It was demonstrated in animal

and scarce human studies that carnitine increases the activities of RC complexes (Huertas et al., 1992, Ruiz-Pesini *et al.,* 2001, Kamaran *et al.,* 2004). In our work, we have found a significant association between free carnitine concentration in the whole cord blood and the mitochondrial enzymatic and CS acitivities (complex I+III).

Premature neonates in comparison with term neonates are born with limited carnitine stores. It may be of importance to notice, that the results of acylcarnitine analyses in the whole blood in neonates may partially rely not only on the gestational age and birth weight but also on the actual haematocrit, especially in neonates with anemia or blood hyperviscosity.

Table 8. Free carnitine (FC), acylcarnitines (AC) and total carnitine (TC) levels and their ratios in plasma from cord blood estimated by radioenzymatic technique (RE) and in the whole cord blood estimated by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) in term neonates (B, D) and premature neonates (A, C)

Group*	A	B	C	D		
n	14	33	27	33		
Material	Cord plasma		Cord blood			
Method	RE		ESI-MS/MS			
					p-value	
Carnitine	(μmol/l)				A:B	C:D
FC	20.8 ± 16.5	6.61 ± 2.4	32.9 ± 6.7	46.87 ± 13.9	<0.01	<0.001
AC	15.6 ± 13.6	3.63 ± 1.46	17.4 ± 3.9	14.49 ± 8.7	<0.01	NS
TC	36.4 ± 19	10.2 ± 3.5	50.3 ± 10	61.36 ± 20.3	<0.01	<0.01
AC/FC	0.78 ± 0.37	0.59 ± 0.31	0.53 ± 0.07	0.31 ± 0.13	NS	<0.001
FC/TC	0.58 ± 0.08	0.65 ± 0.09	0.65 ± 0.03	0.77 ± 0.07	NS	<0.001

* Mean±SD-birth weight (g), gestational. age (weeks): A-1652 ± 728, 32 ± 2.8; B-3485 ± 309, 40 ± 1.4; C-1855 ± 765, 32.6 ± 3.2; D-3485 ± 309, 40 ± 1.4

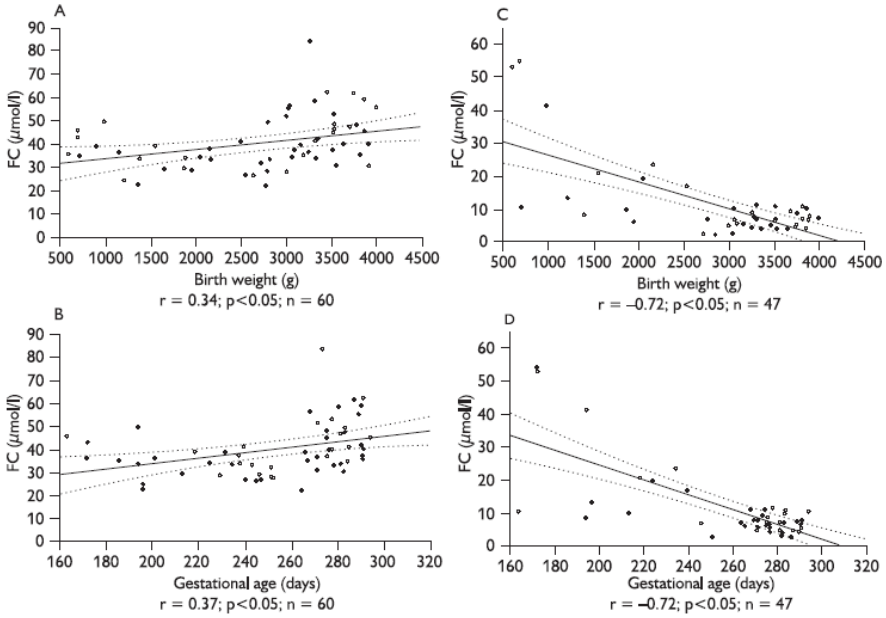

Figure 13. *The correlation between free carnitine (FC) level in the whole cord blood and birth weight (A) or gestational age (B) estimated by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) and the correlation between free carnitine (FC) level in cord plasma and birth weight (C) or gestational age (D) estimated by radioenzymatic technique (RE).*

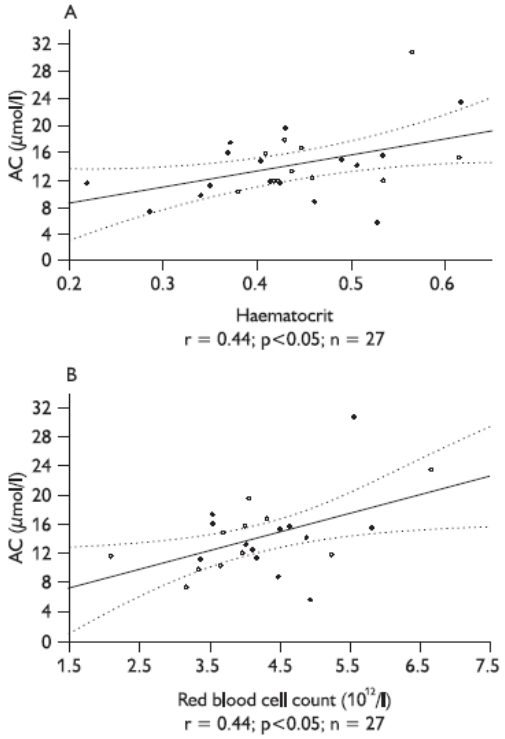

Figure 14. *The relation between acylcarnitines (AC) level in the whole cord blood and haematocrit (A) or red blood cell count (B).*

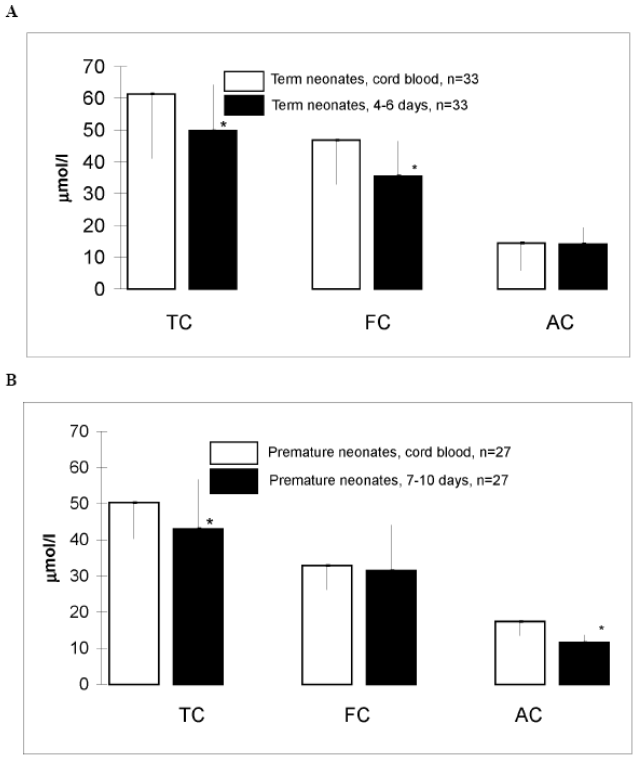

Figure 15*. The comparison between total carnitine (TC), free carnitine (FC) and acylcarnitines (AC) levels in pairs of samples from the cord blood and the blood obtained in term neonates at the postnatal day 4–6 (A) and in premature neonates at the postnatal day 7–10 (B). (* p< 0.05).*

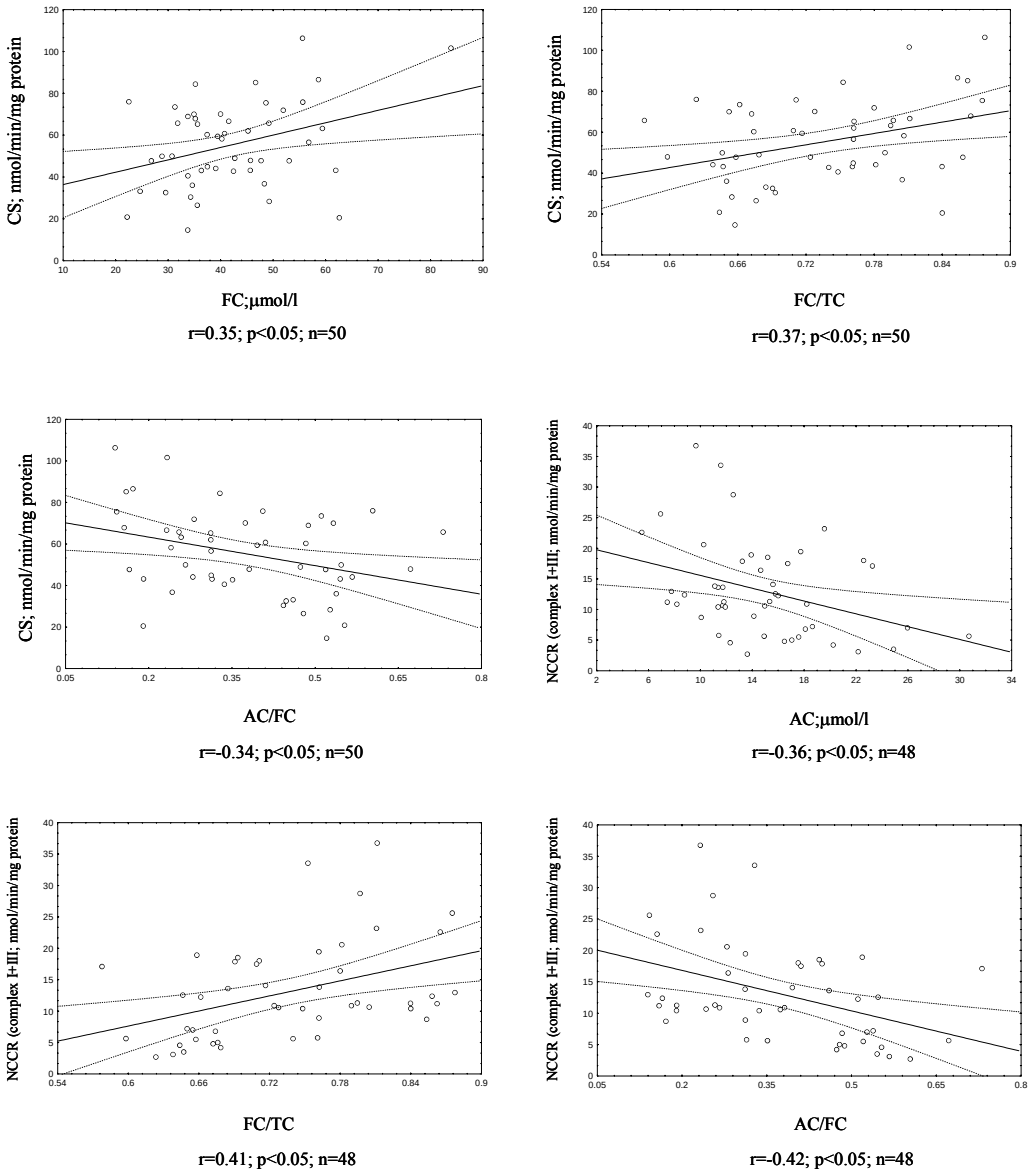

Figure 16. *The relation between acylcarnitines (AC), free carnitine (FC) level and their ratios in the whole cord blood and respiratory chain enzymes activities in isolated lymphocytes obtained from neonates in different gestational age.*

5.5. Polarographic and spectrophotometric studies in isolated muscle mitochondria in permeabilized human muscle fibers

Using spectrophotometric analysis markedly decreased cytochrome *c* oxidase activity was found in three out of six patients (patients 1, 2 and 6). In two patients, a decrease activity of complex I was found (patients 4 and 5) and in two patients (patients 1 and 3) a decrease activity of complex III was ascertained. In patient 1, a combined deficiency of complex III and IV was present. In all patients, the activity of control enzyme citrate synthase was within the reference range. Mitochondrial enzyme activities were further analyzed by oxygen consumption measurements using isolated mitochondria (patients 1 to 3) and saponin skinned muscle fibers (patients 4 to 6). Figure 17 shows the typical oxygraphic curves of isolated muscle mitochondria from control subjects in comparison with patient 1 which allowed

evaluation of the activity of most RC complexes. Our measurements showed a partial disruption of the outer mitochondrial membrane, because the oxygen consumption by isolated mitochondria was activated by exogenous cytochrome *c*. The oxygen consumption without substitution of cytochrome c was by about 20 % lower in comparison with the respiratory rate in the presence of cytochrome *c*. Values of oxygen consumption in isolated mitochondria of patients 1 to 3 shown a decreased respiration with NADH- and FADH-dependent substrates and with ascorbate and TMPD in patients 1 and 2. No changes were found in patient 3. Figure 18 shows typical curves of polarographic measurements with muscle fibers prepared from control and patient biopsies (patient 4). Similarly as with isolated mitochondria, utilization of NADH- and flavoprotein-dependent substrates, activity of cytochrome c oxidase was evaluated. Under these conditions, respiration was not activated by cytochrome c, indicating that the outer mitochondrial membrane was intact. The values of oxygen consumption from our measurements on permeabilized muscle fibers from patients 4-6 clearly identified the complex I deficiency in patient 4, but there were no significant changes in oxygen consumption in patient 5. In patient 6, with an evident COX deficiency recognised by spectrophotometry, the mitochondrial respiration in permeabilized muscle fibers was lower after addition of all substrates.

On the biochemical level, several different approaches are available for determination of the mitochondrial respiratory chain function in the patients with mitochondrial disorders. The activities of respiratory chain complexes may be analyzed spectrophotometrically as dehydrogenases and oxidoreductases or they may be measured polarographically as the oxygen consumption rate after addition of various substrates.

Using spectrophotometic analyses we selected six patients with apparent defects of respiratory chain complexes disorders. Three patients have decreased cytochrome c oxidase activity, two patients decreased activity of complex I and two patients decreased activity of complex III. One of patients has combined defect of complex III and IV.

When we compared the results from the spectrophotometric measurement with the polarographic data in our patients with mitochondrial disorder, we found by both methods defect of cytochrome c oxidase in patient 1 and 2 and normal cytochrom *c* oxidase activity in patient 3. Contrary to spectrophotometric measurements polarographic analysis showed lower rate of NADH- and flavoprotein-dependent substrates utilization in patient 1 and 2 with decreased cytochrome *c* oxidase activity. This discrepancy indicates that capacity of these dehydrogenases cannot be fully utilized when the complex III and/or IV activity is highly depressed.

Polarographic measurements on muscle fibers correlate with those obtained with spectrophotomeric measurements on isolated mitochondria. Defect of cytochrome *c* oxidase was confirmed in patient 6 and no changes of cytochrome *c* oxidase were found in patient 4 and 5. Similarly as in polarographic measurements on isolated mitochondria defect of cytochrome *c* oxidase activity was accompanied by lower utilization of NADH- and flavoprotein-dependent substrates. In patient 4 with pronounced decrease of complex I activity, also the lower utilization of NADH-dependent substrates was found. Thus both oxygraphic analyses in isolated mitochondria and in permeabilized muscle fibers provide similar and corresponding data in diagnostics of mitochondrial defects as spectrophotometric measurements.

The main difference in oxygen consumption studies between isolated muscle mitochondria and permeabilized muscle fibers is the disturbed structural integrity of myocyte and partial disintegration of outer mitochondrial membrane as demonstrated by the activatory effect of added cytochrome c (see Figure 17B). No similar problems occur with saponin-skinned muscle fibers, where activities of mitochondrial enzymes are measured under conditions, which are more close to conditions in intact cell. The technique of the saponin

skinned muscle fibers allows thus to study the mitochondrial respiration "in situ" with the preserved contact of the mitochondria with other cell organelles.

Very important is also correlation of spectrophotometric and polarographic methods when the ratio between COX and other RC complexes is determined. Spectrophotometric measurements showed COX/SQR ratio 18.3, which indicates high reserve capacity of COX (Table 11). However polarographic measurements on isolated mitochondria or muscle fibers under conditions that reflect more the situation in intact cell show that this reserve capacity is in fact much lower (2.5- or 4.8-fold, respectively). This difference is due to activation of COX activity by detergent used in spectrophotometric method. Low excess of COX, observed in muscle fibers with intact mitochondria, are thus in agreement with data observed by measurements of oxygen consumption in different types of cultured cells. These experiments demonstrated only a low excess of cytochrome *c* oxidase capacity, when compared with capacities of various respiratory chain complexes (Villani and Attardi, 1997). Polarographic measurements, indicating that cytochrome *c* oxidase under physiological conditions is not in high excess, it is also in agreement with established role of cytochrome *c* oxidase in control of respiratory chain function (Gnaiger *et al*., 1998, Villani and Attardi, 2000).

The results of our study showed that both methods the spectrophotometry and the oxygraphy provide different but complementary information about the function of respiratory chain complexes and therefore both techniques are important in the diagnostics of mitochondrial disturbances of energy generating system in patients.

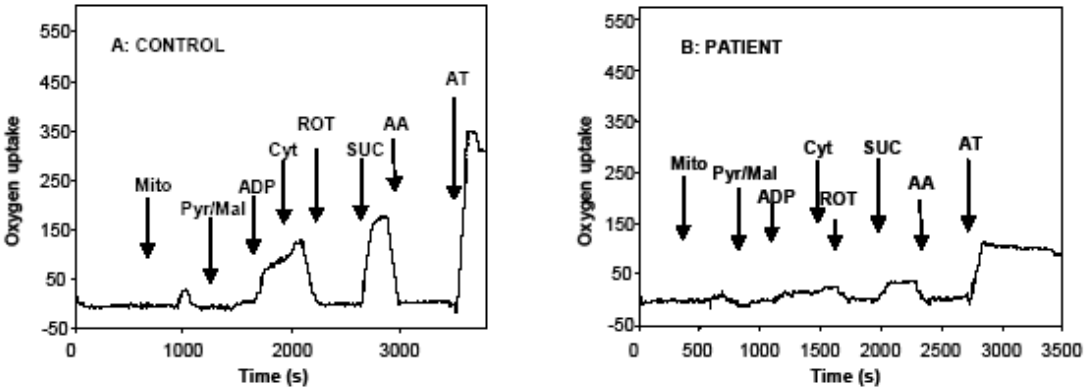

Figure 17. *Determination of oxygen consumption (pmol oxygen/s/mg protein) using multiple substrate-inhibitor analysis in isolated muscle mitochondria from (A) control and (B) patient 1. Additions: Mito: 90 µg protein of isolated muscle mitochondria; Pyr/Mal: 10 mM pyruvate plus 2 mM malate; ADP: 1 mM ADP; Cyt: 5 µM cytochrome c; ROT: 1.25 µM rotenone; SUC: 10 mM succinate; AA: 2.5 µM antimycin A; AT: 0.4 mM ascorbate plus 400 µM TMPD.*

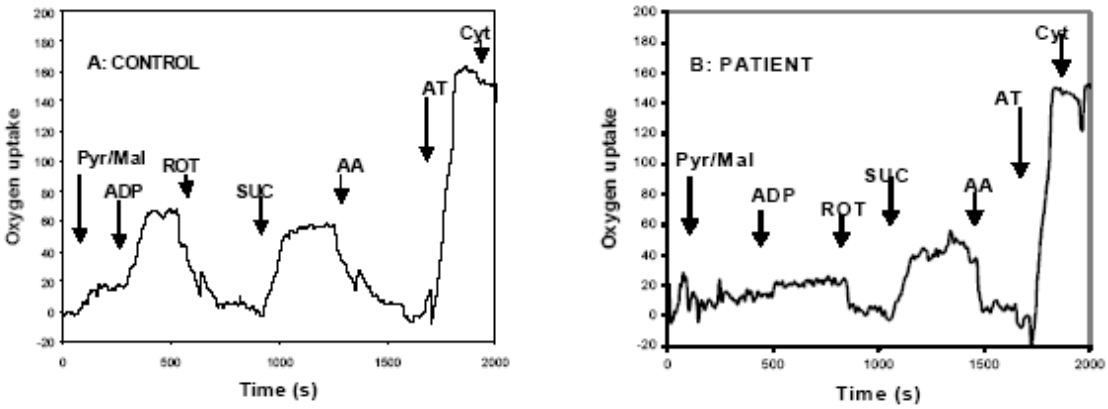

Figure 18. *Determination of oxygen consumption (pmol oxygen/s/mg wet weight) using multiple substrate-inhibitor analysis in permeabilized muscle fibers from (A) control and (B) patient 4. Additions: Pyr/Mal: 10 mM pyruvate plus 5 mM malate; ADP: 1 mM ADP; ROT: 1.25 μM rotenone; SUC: 10 mM succinate; AA: 2.5 μM antimycin A; AT: 2 mM ascorbate plus 500 μM TMPD; Cyt: 5 μM cytochrome c.*

5.6. Mitochondrial energetic metabolism in blood cells

Age dependent differences in the activities of respiratory chain complexes IV and I+III were observed in isolated lymphocytes from umbilical cord blood between premature neonates, term neonates and small children. The results of our study are shown in table 9. The specific activity of respiratory chain complex I+III and CS in isolated lymphocytes from umbilical cord blood in premature neonates were significantly lower in comparison with term neonates. On the contrary, the activity of respiratory chain complex III was higher in premature neonates in comparison with term neonates (Table 9). In the group of 37 controls (at the age between 0.5-2 years) the specific activity of respiratory chain complex IV and CS in isolated lymphocytes from peripheral blood were significanly higher in comparison with term or premature neonates ($p < 0{,}001$ for both parameters). The activity of citrate synthase, used as a marker of mitochondria number per cell, showed clear tendency to increase during childhood. So the differences in activities of RC in our study may result from increasing amount of mitochondria during development. Our data suggest that lymphocytes seem to represent easily available cells, which may be useful for studies of the metabolic changes in children and even in premature neonates. However, the further work is necessary to reduce the rather high amount of blood required for analysis (7 ml).

The reference ranges for activities of the respiratory chain complexes I, II, III, IV and I+III and citrate synthase in isolated platelets were obtained for each of the age groups (0.5-2; 3-9; 10-19; 20-35 years) (Table 10, 11). Isolated platelets contribute only very little to the overall metabolic turnover but because of their easy availability they may serve as a suitable material for analysis of activities of respiratory chain complexes. We suppose that the analysis of activities of respiratory chain complexes can be useful for the enzymatic diagnosis of specific mitochondrial disorders with a generalized affection.

Table 9. The specific activities of respiratory chain complexes and pyruvate dehydrogenase in lymphocytes isolated from umbilical cord in premature and term neonates.

	Lymphocytes				
Gestational age (weeks)		38-42		24-37	
		(nmol/mg/min)			(*p* -values)
NQR	n=37	44.5 + 20.2	n=14	36.6 + 15.5	NS
NCCR	n=37	15.3 + 8.3	n=16	8.3 + 5.5	<0,01
SQR	n=35	11.2 + 5.1	n=17	8.5 + 2.6	NS
QCCR	n=35	28.3 + 9.7	n=9	38 + 23	<0,05
COX	n=38	22.45 + 5.5	n=14	22.5 + 7.2	NS
CS	n=38	61.04 + 17.9	n=17	44.1 + 17.4	<0,01
COX/CS	n=38	0.4 + 0.16	n=14	0.62 + 0.3	<0,01
PDH	n=35	0.57 + 0.3	n=13	0.55 + 0.3	NS

NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), NCCR: NADH-cytochrome *c* oxidoreductase (complex I+III), SQR: succinate coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome c oxidoreductase (complex III), COX: cytochrome c oxidase (complex IV), PDH: pyruvate dehydrogenase , CS: citrate synthase. *p*-value of <0.05 was considered significant. NS: nonsignificant. Control group of 37 small children at the age of 0.5-2 years was used for comparison between activity of respiratory chain complex IV and CS. Specific activities of our control group of small children were as follow- COX: 35.5 ± 13.7 nmol/mg/min, CS: 78.1 ± 18.2 nmol/mg/min, COX/CS: 0,46 ± 0.17, respective.

Table 10. Activities of respiratory chain complexes I, II, III, IV and I+III and citrate synthase in isolated platelets in 161 children, adolescents and young adults (mean ± SD).

	Platelets			
Age (years)	0.5 – 2	3 – 9	10 – 19	20 – 35
n	32	50	39	40
		(nmol/mg/min)		
NQR	34.5 ± 16.2	35.6 ± 16.2	39.1 ± 15.7	36.4 ± 16.0
NCCR	15.8 ± 7.2	17.6 ± 7.3	16.3 ± 4.7	15.6 ± 6.7
SQR	8.2 ± 2.9	9.6 ± 3.4	10.6 ± 3.3	9.4 ± 4.0
QCCR*	16.4 ± 6.6	14.5 ± 4.0	19.2 ±7.7	15.2 ± 5.3
COX	21.6 ± 5.2	21.5 ± 5.4	21.6 ± 4.7	21.0 ± 4.7
CS	71.5 ± 13.4	76.4 ± 17.0	76.4 ± 15.6	81.7 ± 18.5

NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), NCCR: NADH-cytochrome c oxidoreductase (complex I+III), SQR: succinate coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome c oxidoreductase (complex III), COX: cytochrome c oxidase (complex IV), CS: citrate synthese. *number of investigations of complex III in individual age groups n = 6 – 38.

Table 11. The ratios between the activities of individual respiratory chain complexes and citrate synthase in 161 children, adolescents and young adults in four age groups (mean ± SD).

	Platelets			
Age (years)	0.5 – 2	3 – 9	10 – 19	20 – 35
n	32	50	39	40
NQR/CS	0.49 ± 0.22	0.48 ± 0.24	0.52 ± 0.20	0.47 ± 0.22
NCCR/CS	0.22 ± 0.10	0.24 ± 0.13	0.23 ± 0.10	0.19 ± 0.08
SQR/CS	0.12 ± 0.04	0.13 ± 0.05	0.14 ± 0.06	0.12 ± 0.05
QCCR/CS	0.26 ± 0.13	0.20 ± 0.05	0.24 ± 0.09	0.20 ± 0.08
COX/CS	0.30 ± 0.06	0.28 ± 0.06	0.29 ± 0.08	0.26 ± 0.06

NQR: NADH-coenzyme Q_{10} oxidoreductase (complex I), NCCR: NADH-cytochrome c oxidoreductase (complex I+III), SQR: succinate coenzyme Q_{10} oxidoreductase (complex II), QCCR: coenzyme Q_{10}-cytochrome c oxidoreductase (complex III), COX: cytochrome c oxidase (complex IV), , CS: citrate synthase. *number of investigations of complex III in individual age groups n = 6 – 38.

6. CONCLUSIONS

Our studies were focused on specific properties of mitochondrial energetic metabolism in term and premature neonates.

a) Age dependent differences in the activities of respiratory chain complexes and pyruvate dehydrogenase complex were observed during fetal development, early postnatal period and childhood. Electrophoretic and Western blot analyses in muscle mitochondria revealed decreased protein amount of respiratory chain complexes and a lower amount of subunits of pyruvate dehydrogenase. These changes may reflect a lower mitochondrial proteins synthesis in very premature neonates and/or an increase in mitochondrial proteins degradation due to stress or sepsis. In addition, the pyruvate dehydrogenase activity was significantly lower in premature neonates with severe hyperlactacidemia. Our results indicated that monitoring of glucose level, frequent controls of lactate and pyruvate levels and their ratio are important in critically ill premature neonates.

b) Mitochondrial respiratory chain complexes are present in placenta at proportion similar to other tissues with exception of glycerophosphate dehydrogenase (mGPDH) which is expressed at a very high rate. Using fluorometric probe dichlorodihydrofluorescein diacetate we found that placental mitochondria display high rate of glycerophosphate-dependent hydrogen peroxide production. This was confirmed by oxygraphic detection of glycerophosphate-induced, KCN- or Antimycin *A*-insensitive oxygen uptake. Our results indicate that mGPDH should be considered as an additional source of reactive oxygen species participating in induction of oxidative stress in placenta.

c) Our study on premature neonates endorses the hypothesis that premature neonates are born with limited carnitine stores. It may be of importance to notice, that the results of acylcarnitine analyses in the whole blood in neonates may partially rely not only on the gestational age and birth weight but also on the actual haematocrit, especially in neonates with anemia or blood hyperviscosity. Low cord blood carnitine pool and low carnitine tissue stores in premature neonates may futher diminished ATP production and influence postnatal adaptation.

d) We may conclude from our experiments of detection of mitochondrial disorders using polarographic and spectrophotometric methods, that both methods provide useful and complementary data about mitochondrial energetic function. Whereas spectrophotometric data are better for evaluation of maximal enzyme activities of mitochondrial enzyme complexes, polarographic data better reflect enzyme activities in cells with mitochondrial defects under *in situ* conditions.
e) The reference ranges for activities of the respiratory chain complexes and citrate synthase in isolated platelets and isolated lymphocytes were established for neonates. Our data suggest that lymphocytes and platelets seem to represent easily available cells, which may be used for the enzymatic diagnosis of specific mitochondrial disorders with a generalized affection and for studies of the metabolic changes in children and even in premature neonates.

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