Placental Tissue as Model for Pilot Study Focused on RNA Analysis from Human Foetal Tissue

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Abstract: Early neonatal adaptation to extrauterine life is i.a. dependent on effective mitochondrial biogenesis during foetal development. Understanding of mitochondrial biogenesis is limited, because only scarce data are available from prenatal studies including RNA analyses in human foetal tissues. Aims of the study were focused on the factors affecting RNA quality in human placental tissue (HPT) including temperature, time period before HPT freezing and the Apgar score. In addition, optimal reference genes for mRNA quantification by real-time PCR in HPT were studied. Samples of HPT were obtained after the birth of 20 term neonates. Seven HPT were used for the time-course study of RNA degradation in two different temperatures (0 °C and 24 °C). Various instruments NanoDrop (NanoDrop Technologies), Experion (Bio-Rad Laboratories), Agilent 2100 Bioanalyzer (Agilent Technologies) were used for analysis of RNA integrity, purity and yield. Identification of suitable reference genes was achieved by analysing six candidate genes (*ATP5O, SDHA, TBP, HPRT, PMBS, ATP6*) for their expression stability (GeNorm application). The results showed that the HPT samples for RNA analyses must be frozen immediately after birth in –80 °C or stored at 0 °C maximally for 1 hour. The reference genes *ATP50* and *SDHA* were the most stable for mRNA quantification in HPT. Human placenta represents easily obtainable source of foetal tissue for studies concerning mitochondrial biogenesis. We demonstrated that the critical limit for optimal storage and handling of HPT are the temperature and the time period before freezing of the samples.

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Introduction

Postnatal switch of glycolytic to oxidative metabolism is of crucial importance for all mammalian neonates and it is essential for successful adaptation to extrauterine life. Recruitment of mitochondrial oxidative phosphorylation in the tissues of neonates reflects changes in hormonal status and the postnatal shift from glucose to glucose and fats as the major energy sources (Ferre et al., 1986). Postnatal adaptation of the neonate to extrauterine life is i.a. dependent on effective mitochondrial biogenesis during foetal development.

Mitochondrial biogenesis can be simply defined as the growth and division of pre-existing mitochondria. It is affected both by genetic background and environmental factors including cell division and renewal, tissue differentiation, caloric availability or restriction, oxidative stress, cold exposure, exercise, etc. The complexity of mitochondrial biogenesis involves changes in the expression of more than 1,000 genes and the cooperation of two genomes (Lenka et al., 1998). Mitochondrial DNA (mtDNA) is a circular double-stranded molecule of approximately 16,600 base pairs encoding for 13 proteins of the approximately 90 different proteins present in the oxidative phosphorylation system (OXPHOS) (Papa, 1996). All other components of the OXPHOS are encoded by nuclear genes and are imported to mitochondria via specialized import systems (Chacinska et al., 2002; Truscott et al., 2003).

Better understanding for the mitochondrial biogenesis regulation and function may improve the care of premature neonates or critically ill newborns (Arnon et al., 2002; Honzik et al., 2008). However, understanding of mitochondrial biogenesis during human foetal development is limited due to scarce data from the prenatal studies. Moreover no study exists about foetal tissue handling and further processing especially RNA analysis.

Human placenta represents easily obtainable source of human foetal tissue. This study aims to evaluate appropriate options for handling and storage of human placental tissue (HPT) samples with respect to following RNA analysis. Moreover, we tried to evaluate basic rules for manipulation with foetal tissues.

Material

Samples of HPT were obtained from foetal part of placenta after birth of 20 term neonates (10 boys and 10 girls) with birth weight 3,477 ± 573 g (range 2,610–4,730 g) and gestational age 40 ± 1 weeks (range 37–42 weeks). Nine neonates were delivered by the Caesarean section, the other eleven vaginally. In five neonates the early postnatal adaptation was complicated because of the mild perinatal asphyxia with Apgar score (3–7 in the fifth and tenth minutes).

Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Medical Ethics

at the General University Hospital in Prague. Informed consents were obtained from women prior to taking tissue samples.

Methods

The HPT samples were immediately frozen in liquid nitrogen after washing by physiological buffer, 3–6 samples were frozen according to the time-plan (1. interval = 1 hour, 2. interval = 2 hours and 3. interval = 3 hours) for time-course study (the part of them was kept in 0 °C and the other part was kept in 24 °C).

RNA was isolated from HPT samples by TriReagent solution (MRC). The isolation was performed according to recommended protocols of the manufacturer.

Total RNA was treated by DNase I (Ambion) according to manufacturer protocol. The quality and the yield of total RNA were checked by Agilent Bioanalyzer 2100 (Agilent Technologies) and NanoDrop 1000 (Thermo Scientific). Electrophoresis was used to check RNA integrity and purity and was prepared according to Masek et al. (2005). Analysis of RNA integrity number was used for HPT samples.

The RNA integrity number (RIN) is a software tool designed for estimation of total RNA samples integrity. Using this tool, sample integrity is not determined by the ratio of ribosomal bands, but by the entire electrophoretic trace of the RNA sample. This includes the presence or absence of degradation products (Agilent Technologies: www.home.agilent.com). The RIN software algorithm allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact.

One thousand nanograms of total RNA was transcribed to cDNA using Superscript III Reverse Transcriptase (Invitrogen), FSB (First Strand Buffer) (Invitrogen), DTT (dithiothreitol) (Invitrogen) and Oligo-dT primers (Promega) (thermal conditions, Table 1). Obtained cDNA was stored at –20 °C.

Table 1 – Reverse transcription – reaction mixture and thermal cycling conditions

Mixture 1 (10 µl)	Final concentration in reaction (20 µl)	Thermal cycling conditions		
		step	temperature	time
RNA template	1–2 µg	1. initial denaturation	72 °C	2'
Oligo dT primer	0.5 µg			
Mixture 2 (10 µl)	Final concentration in 1 reaction (20 µl)			
FSB	1×			
DTT	10.0 mM	2. reverse transcription	42 °C	50'
dNTP	0.5 mM	3. enzyme inactivation	70 °C	15'
RNAsin	20 U			
SuperScript III	100 U			

FSB – First Strand Buffer (Invitrogen); DTT – dithiothreitol (Invitrogen); dNTP – deoxynucleotide triphosphate (Promega); RNAsin (Promega)

Fragments of the *MECP2* gene – 378 bp fragment which involves the exon-exon boundaries and 1134 bp fragment which includes intron – were applied as a control of genomic DNA (gDNA) contamination in placenta RNA resp. cDNA samples (in case of contamination, there were both fragments found) (Table 2).

GeNorm application, a Microsoft Excel programme available at: http://medgen.ugent.be/wjvdesomp/genorm/ was used to test which reference genes are the most suitable for HPT. The reference genes were selected from group of six candidate genes (*ATP6* (ATP synthase 6), *ATP5O* (ATP synthase 5O), *SDHA* (succinate dehydrogenase complex, subunit A), *HPRT1* (hypoxanthine phoshoribosyltransferase 1), *TBP* (TATA box binding protein 1), *PSMB6* (protesome beta 6 subunit)) according to the value of expression stability (M). Determination of M is that the lowest M value is for the most stable gene and the gene with the highest instability has the highest M value (Vandesompele et al., 2002). Real-time PCR amplification was performed as described in Pejznochova et al. (2010).

Results and Discussion

Important requirement for successful analyses using real-time PCR is equal RNA quality in all samples. In our study we analyzed the impact of temperature, time period and Apgar score on RNA quality and integrity in human placental tissue. Especially low Apgar score with increased lactate level in umbilical blood increases the probability that the foetus passed hypoxemia. The hypoxemia might have some negative impact on appropriate quality of RNA for objective study.

Generally, the significant negative correlation was observed between RNA yield and RNA purity of the HPT samples ($r = –0.61$, $p<0.01$; $y = 12845.19 – 5332.21x$). There was also significant positive correlation between RNA yield and sample weight ($r = 0.46$, $p<0.05$; $y = 207.87 + 0.06x$). The individual data (RNA yield, RNA purity and RNA integrity) of each sample are shown in Table 3.

In the time course study the correlation was found between RNA yield and RNA purity for 0 °C in the first ($r = –0.94$, $p<0.05$; $y = 22677.73 – 10181.37x$) and

Table 2 – PCR reaction mixture and thermal cycling conditions for MECP2 fragment – control of gDNA contamination in RNA (cDNA) samples

PCR mixture	Final concentration in reaction (25 µl)	PCR conditions		
		step	temperature	tim
DNA template	50–100 ng	1. initial denaturation	95 °C	2'
CombiPPPMaster Mix	500 µg/ml	2. denaturation	95 °C	30"
primer forward	1×	3. annealing	60 °C	30"
primer reverse	10 mM	4. extension	72 °C	1'
		30 times repeating steps 2 to 4		
		5. final extension	72 °C	10'

the second ($r = –0.8384$, $p<0.05$; $y = 28653.18 – 13366.12x$) intervals (data not shown). It means that the higher is RNA yield the lower is the RNA purity. The low Apgar score did not affect the RNA yield. Contrary to the yield, the RNA purity of low Apgar samples was the worst in our group of samples.

Table 3 – Characteristics of RNA samples from HPT

Sample No.	RNA yield (ng/μ)	RNA purity	Tissue weight (mg)	RIN
1	**4290**	**1.62**	**590**	**5.3**
2	**4472**	**1.92**	**440**	**4.8**
3	**4411**	**1.52**	**830**	**5.2**
4	**4464**	**1.98**	**680**	**5.4**
5	655	1.96	360	6.9
6	2105	1.98	100	4.1
7	4155	1.72	470	4.7
8	4286	1.63	550	4.9
9	3407	1.92	430	5.7
10	**4401**	**1.52**	**300**	**N/A**
11	4223	1.70	270	5.7
12	2400	1.97	290	6.3
13	453	1.88	360	7.2
14	1655	1.97	190	6.3
15	775	1.93	470	5.4
16	2958	1.95	210	4.9
17	1819	1.97	300	5.9
18	3518	1.88	470	6.4
19	3814	1.83	270	3.0
20	2204	1.99	160	3.8

The numbers in bold marks the placental samples from children with low Apgar score (3–7 at the fifth minute). Samples Nos. 1–9 are placental samples from children born with Caesarean sections and Nos. 10–20 are placental samples from spontaneously born neonates. RIN – RNA integrity number

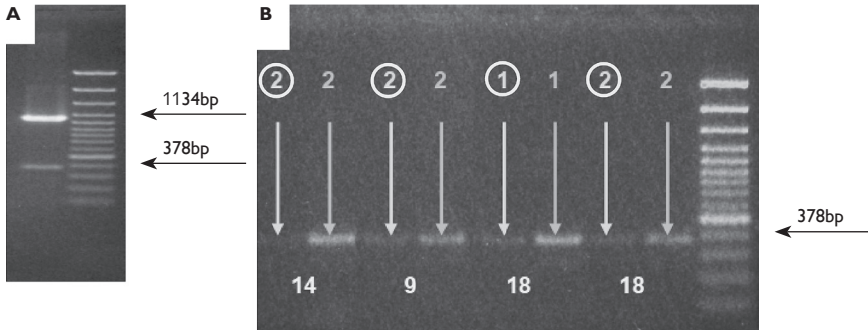
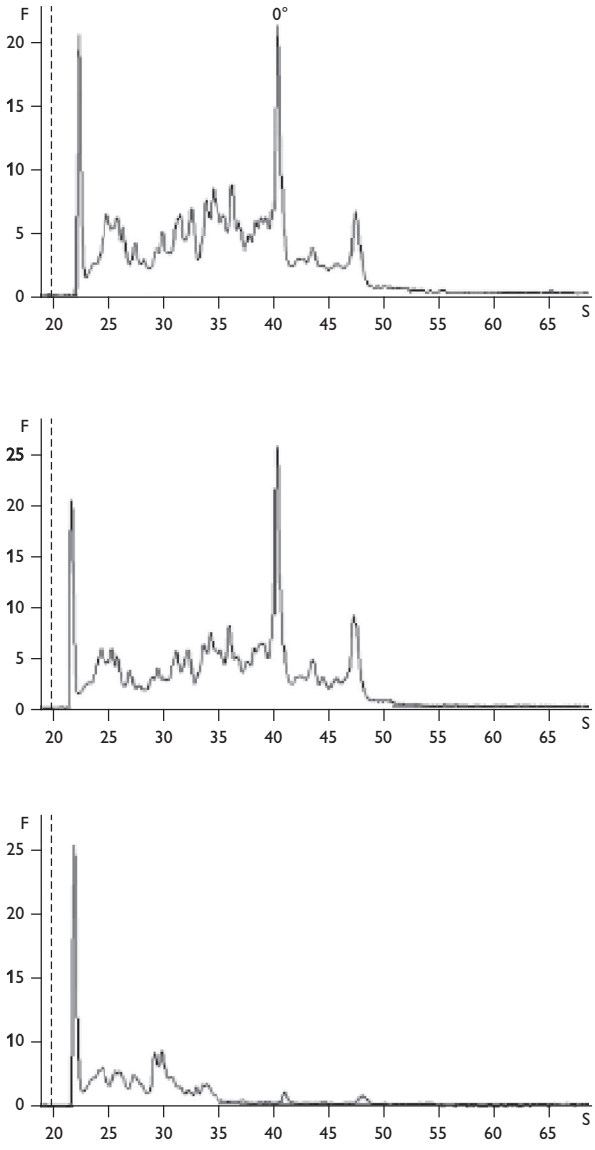


Figure 1 – Analysis of gDNA contamination in cDNA samples and the effect of different temperatures and the time periods on cDNA amplification. A – sample No. 14 with added gDNA (two fragments of MECP2 – 1134 bp from gDNA and 378 bp from transcribed RNA respectively cDNA); B – amplification of MECP2 fragment (378 bp) in the samples (Nos. 9, 14, 18) of the first and the second intervals (the numbers above arrows mark intervals, the numbers in circle show 24 °C and the numbers without circle show 0 °C).

All our HPT samples were found without gDNA contamination. The longer *MECP2* gene fragment (1143 bp) was amplified from gDNA and therefore it was marker of gDNA contamination in RNA sample (Figure 1A). In all samples only shorter *MECP2* gene fragment (378 bp) was amplified (Figure 1B), which means that RNA resp. cDNA was pure. On the other hand, significant differences were found in the intensity of shorter *MECP2* gene fragment on electrophoresis gel (Figure 1B). These differences correspond with the temperature (0 °C or 24 °C)

A



and also with time period before freezing of the samples. RNA from tissue sample which was placed in the lower temperature (0 °C) provided better PCR result. Moreover the shorter time interval (1 hour) showed still better PCR result. It means that the intensity of *MECP2* amplification reflected the RNA integrity, which was changed during intervals and due to the temperature.

The role of time period before freezing was crucial as showed the analysis of the RNA integrity. We found, that the longer is the time period before freezing the

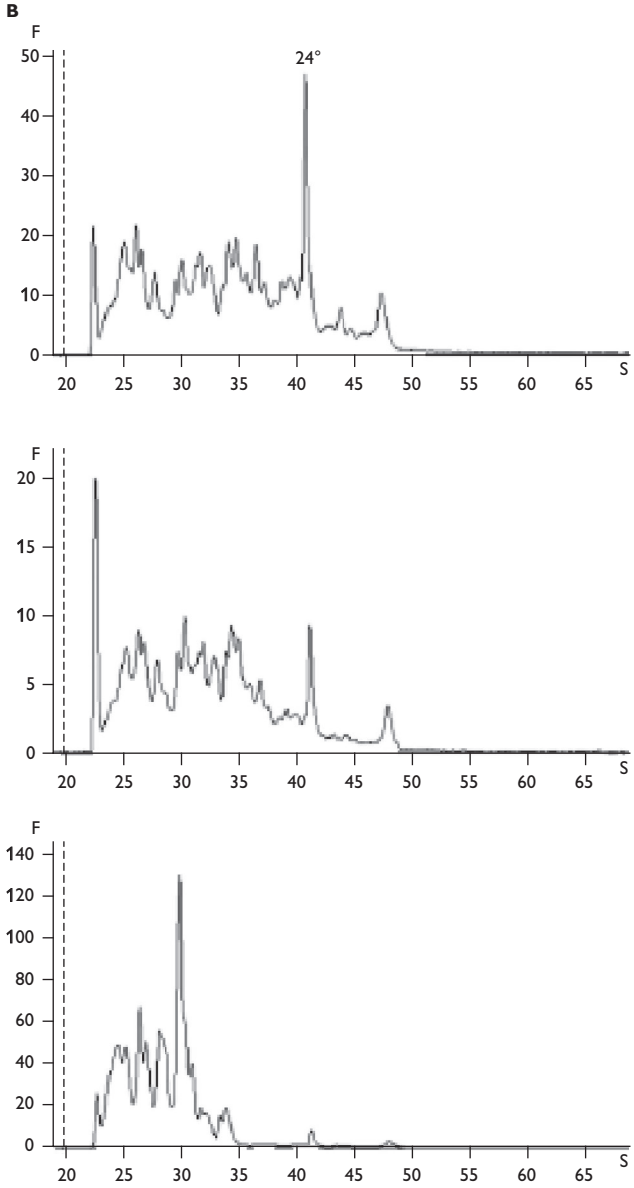


Figure 2 – Degradation of RNA during the time periods in different temperatures.
The 18S rRNA and 28S rRNA peaks (40s and 48s) of sample No. 12 in the three time intervals and two different temperatures (0 °C and 24 °C). When peaks disappear it means that the RNA integrity decreases. RINs were 5.8/5.3 (after 1 hour), 5.8/4.8 (after 2 hours), 2.5/2.3 (after 3 hours).

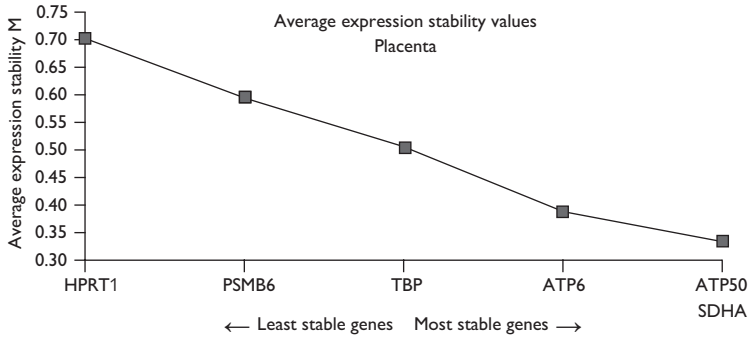


Figure 3 – GeNorm analysis – selection of reference genes.
The six genes (ATP6, ATP5O, SDHA, HPRT1, TBP, PSMB6) were tested in placental tissue (n=12).

lower is the RNA integrity similarly to results obtained in *MECP2* analysis. Other important factor for the RNA integrity was temperature. According to the RIN the integrity of samples which were kept in 0 °C (first hour: 5.4 ± 0.9; second hour: 5.0 ± 1.5) decreased slower then the integrity of those samples kept in 24 °C (first hour: 4.4 ± 1.1; second hour: 3.5 ± 0.9). This dependence was invalid for samples which were kept for three hours in 0 °C respectively in 24 °C (third hour: 2.6 ± 0.1; 2.4 ± 0.1). It was probably influenced by the long-time period before freezing and the small number of analysed samples in the third interval. In summary, storage of the tissue samples in 0 °C kept the RNA integrity relatively constant for 2 hours, meanwhile it was degraded more rapidly in 24 °C (Figure 2). The average RIN of 20 HPT samples was 5.5 ± 1.0.

Apart from RNA quality, the choice of a proper set of reference genes for accurate normalization is another crucial factor with a profound impact on the reliability of the obtained gene expression levels (Perez et al., 2000). Therefore the reference genes for HPT were also determined. In our study in placental tissue, *ATP50* and *SDHA* were the most stable expressed genes with M = 0.340 (Figure 3). Similar result for HPT was shown in study of Meller et al. (2002).

Conclusion

According to our results, the important factors for handling, storage and acquirement of the best RNA quality of HPT are the temperature and the time period before freezing of samples. Therefore the proper conditions are the storage in 0 °C for maximally 1 hour or immediate freezing in –80 °C.

Apgar score could be used as potential marker of RNA quality of HPT. Lower Apgar score increases the probability of impurities in RNA from HPT.

The results of our study may be essential for research of mitochondrial biogenesis during human foetal development (Pejznochova et al., 2010). Proper manipulation with tissues after biopsies is necessary especially for optimal RNA analyses and for the reliability of achieved results.

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