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Unstable Expression of Commonly Used Reference Genes in Rat Pancreatic Islets Early after Isolation Affects Results of Gene Expression Studies

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Abstract

The use of RT-qPCR provides a powerful tool for gene expression studies; however, the proper interpretation of the obtained data is crucially dependent on accurate normalization based on stable reference genes. Recently, strong evidence has been shown indicating that the expression of many commonly used reference genes may vary significantly due to diverse experimental conditions. The isolation of pancreatic islets is a complicated procedure which creates severe mechanical and metabolic stress leading possibly to cellular damage and alteration of gene expression. Despite of this, freshly isolated islets frequently serve as a control in various gene expression and intervention studies. The aim of our study was to determine expression of 16 candidate reference genes and one gene of interest (F3) in isolated rat pancreatic islets during short-term cultivation in order to find a suitable endogenous control for gene expression studies. We compared the expression stability of the most commonly used reference genes and evaluated the reliability of relative and absolute quantification using RT-qPCR during 0–120 hrs after isolation. In freshly isolated islets, the expression of all tested genes was markedly depressed and it increased several times throughout the first 48 hrs of cultivation. We observed significant variability among samples at 0 and 24 hrs but substantial stabilization from 48 hrs onwards. During the first 48 hrs, relative quantification failed to reflect the real changes in respective mRNA concentrations while in the interval 48–120 hrs, the relative expression generally paralleled the results determined by absolute quantification. Thus, our data call into question the suitability of relative quantification for gene expression analysis in pancreatic islets during the first 48 hrs of cultivation, as the results may be significantly affected by unstable expression of reference genes. However, this method could provide reliable information from 48 hrs onwards.

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Introduction

Pancreatic islet transplantation into the portal vein of diabetic recipients represents a promis-ing therapeutic alternative for the treatment of insulin-dependent diabetes mellitus since its re-launch by the Edmonton group in the year 2000 [1]. Despite the method has been considerablyimproved, its overall efficiency still needs to be enhanced. One of the possible approaches is to support islet viability during the isolation procedure and over subsequent steps by short-termsilencing of specific genes, i.e. genes involved in apoptotic pathways, in coagulation, etc. This process requires manipulation and *in vitro* cultivation of isolated islets for several hours or days, whereby the precise quantification of target gene expression during all phases of islet iso-lation, preservation, and transplantation is the ultimate condition of success.

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) is a well-established method for quantifying mRNA in biological samples. Its benefits include high sen- sitivity, a large dynamic range, and the potential for high-throughput and accurate expressionprofiling of selected genes. Despite being a powerful technique, RT-qPCR is an indirect methodprone to errors that are easily introduced through a number of steps during the experiment (sample handling, amount of starting material, RNA extraction, nucleic acid quality, enzymaticefficiencies, primer quality and characteristics), thereby increasing the risk of misinterpreting results [2,3]. Therefore, reliable normalization is required for the accurate determination of gene expression level with minimal experimental error. The most common method for nor- malizing cellular mRNA data is the use of reference genes (RGs) as internal controls; however, it is essential that the expression of selected gene(s) is stable and not affected by the experimen-tal conditions [2–5]. Furthermore, an adequate control sample is necessary to estimate any changes or shifts in gene expression related to experimental interventions.

Isolation of pancreatic islets from exocrine tissue is complicated procedure which is critical for the survival of endocrine cells. Nevertheless, this process also exerts severe mechanical and metabolic stress on the islets and can lead to cellular damage and alteration of gene expression.In line with this, Marselli et al. [6] report in their study that immediately after isolation, 4560 genes were up-regulated and 1226 genes down-regulated in isolated islets compared with dataobtained by microdissection from intact pancreatic tissue. Negi et al. [7] report that in freshlyisolated islets, genes involved in mRNA catabolism are highly (21.9-fold) up-regulated, while genes associated with transcription are down-regulated (2.5-fold). Despite of this, both freshly isolated rodent and human islets frequently serve as controls in various gene expression and intervention studies, irrespective of the possible changes in gene expression due to the isolationstress [8–11].

Some conventional RGs, such as 18S rRNA, GAPDH, and β-actin, are classically consideredas constitutively expressed in different tissues and often automatically used as normalizers because of their robust expression. However, there is a growing body of evidence showing that expression levels of these "classic" RGs can vary extensively due to diverse experimental condi- tions [3–5], thus making their routine use as internal controls inadequate for precise quantita- tive normalization because of the large measurement error [3–5,12,13], particularly in pancreatic islets [11,14]. Thus, appropriate validation step is a crucial requirement for avoidingmisinterpretations of study findings, since the RGs selection appears to be highly specific for a particular experimental model [2].

To our best knowledge, no complex comparison study of frequently used RGs is available for islet grafts as well as evaluation of RG expression stability in the early period after isolation.Hence, we determined gene expression of the 16 most commonly used RGs (18S rRNA, Actb, Arbp, B2m, Gapdh, Gusb, Hmbs, Hprt, Pgk1, Ppia, Ppib, Rplp2, Tbp, Tfrc, Ubc, and Ywhaz) [15,16] and one gene of interest (tissue factor, F3) in freshly isolated rat pancreatic islets and

after 24, 48, 72, 96 and 120 hrs of cultivation, in order to compare gene expression stability andto identify reliable RGs suitable for gene expression quantification.

Methods

Isolation and cultivation of rat pancreatic islets

All experiments were performed on Brown Norway rats (male, ~300 g) purchased from Charles River Laboratories, Germany, and were approved by The Animal Care Committee of the Institute for Clinical and Experimental Medicine and Ministry of Health of Czech Republic(Permit Number: 34/2012; 83/2013). Animals were held according to the European Conven- tion on Animal Care in conventional breeding facility with 12/12 hrs light/dark cycle and freeaccess to food pellets and water. A total of 38 rats were used in this study. Pancreatic islets wereisolated using collagenase digestion followed by separation in a discontinuous density gradient,as previously described [17]. Briefly, pancreata of deeply anesthetized rats (Narketan/Dexdo- mitor 4:1, 0.065 ml/100 g b. wt.) were cannulated through the bile duct and filled with 15 ml of collagenase solution (Sevapharma, 1 mg/ml). After excision of pancreas, animals were sacri- ficed by exsanguinations. Excised pancreata were incubated for 18 min at 37°C with moderate shaking. Collagenase digestion was stopped by several cold Hank's Balanced Salt Solution (sup-plemented with 1% FBS) washing steps and the tissue suspension was filtered through a
500 μm mesh. Pancreatic islets were separated using Ficoll (Sigma Aldrich) discontinuous den-sity gradient (1.108 g/ml, 1.096 g/ml, 1.069 g/ml, 1.037 g/ml) yielding in 600–700 purified isletsper rat. Isolated islets were harvested immediately after isolation or cultivated in CMRL-basedculture medium supplemented with 10% FBS, 10 mM HEPES, 2 mM Glutamax, 100 U/ml Pen-icillin, and 100 μg/ml Streptomycin. Islets were harvested after 24, 48, 72, 96 and 120 hrs of cul-tivation and also in the period 0–24 hrs every 3 hrs. Samples of hand-picked islets free of exocrine tissue were frozen in liquid nitrogen and stored at -80°C until the RNA isolation.
Pooled samples including islets of all sizes were used, i.e. islets with more than 150 um in diam-eter were not excluded from the study. The representative sample of isolated islets is shown on S1 Fig. Viability of isolated pancreatic islets was assessed using propidium iodide/acridine orange staining and ranged from 85 to 95% throughout the whole experiment. The insulin con-tent in different time points of cultivation was proved by dithizone staining.

Glucose-stimulated insulin secretion (GSIS) test

The ability of stimulated insulin secretion of freshly isolated islets and after 6, 24, 48, 72, 96 and 120 hrs of cultivation was determined using glucose-stimulated insulin secretion test. Prior the assay, islets (20 islets per well) were placed on polyester membranes with 8 μm pores (Transwell cell culture inserts, Corning) in six well plate with culture media. The GSIS assay medium consisted of Krebs-Ringer bicarbonate buffers equilibrated with 5% CO_2 at 37°C, and supplemented with either 3 mM (low) or 22 mM (high) glucose. All incubations were carried on at 37°C and 5% CO_2, in the volume of 4 ml. Islets were equilibrated for 15 minutes in low glucose GSIS assay medium. Then, glucose-stimulated insulin secretion was tested over 3 x 60 min at three subsequent concentrations of glucose (low, high, and low again). After each incubation period, the insulin content in every well was assessed by radio- immunoassay method (Insulin Coated Tube RIA Kit, MP Biomedicals). After the assay, isletswere washed with PBS, collected and lyzed using proteinase K, EDTA and SDS (Sigma Aldrich). Total DNA content in each islet aliquot was determined using dsDNA specific assay, PicoGreen kit (Invitrogen).

RNA isolation and reverse transcription

RNA was isolated using RNeasy Plus Mini Kit (Qiagen) including a column for elimination of genomic DNA. RNA concentration was determined using Qubit RNA HS Assay Kit (Life Technologies). RNA purity was assessed using NanoDrop 2000 UV-Vis Spectrophotometer asthe 260 nm/280 nm absorbance ratio. In order to prove the RNA quality, the RNA Integrity Number (RIN) was measured in a separate experiment using Agilent RNA 600 Nano Kit (Agi-lent Technologies). Based on electrophoretic separation, this method provides sizing, quantifi-cation and quality control of RNA. Also, the average RNA yield for one islet was calculated in different time points of cultivation. The fixed amount of RNA (250 ng) was reversely tran- scribed to cDNA with High-Capacity RNA-to-cDNA Kit (Life Technologies) according to manufacturer´s instructions and stored at -20°C until the gene expression analysis.

RT-qPCR

In the first experiment, the expression of 16 candidate RGs (18S rRNA, Actb, Arbp, B2m, Gapdh, Gusb, Hmbs, Hprt, Pgk1, Ppia, Ppib, Rplp2, Tbp, Tfrc, Ubc, Ywhaz) normalized to thesame RNA input was determined using TaqMan® Rat Endogenous Control Array 384-well micro fluidic cards on AbiPrism 7900 (Life Technologies). Full names, IDs, function and loca-tion of all genes can be found in S1 Table. In the second experiment, absolute quantification ofGapdh, Ppia, and F3 was performed. Specific primers for these genes (F3: 5^0-GATAAAGACAGTGACCAGGAACA-3^0, 5^0-CTAACCACAAGAGCCCAGAA-3^0; Gapdh: 5^0-GTAACCAGGCGTC CGATAC-3^0, 5^0-TCTCTGCTCCTCCCTGTTC-3^0; Ppia 5^0-CCATTATGGCGTGTGAAGTC-3^0, 5^0- GCAGACAAAGTTCCAAAGACAG-3^0) (IDT) were used to amplify the defined segment of the respective mRNA. Amplified segments were separated on 2% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen). DNA concentration was determined using Qubit DNA Assay Kit (Life Technologies). Purified fragments were used to construct the calibration curves.As a template for segment amplification, total RNA isolated from islets after 72 hrs of cultiva-tion was used. Absolute quantification of F3, Gapdh and Ppia gene expression was performedon ViiA™ 7 Real-Time PCR System (Applied Biosystems) using PrimeTime® qPCR probe- based gene expression assays (IDT).

Data analysis and statistics

Gene expression data were analyzed using RQ Manager and ViiA™ software. Absolute cDNA quantity was calculated from the appropriate calibration curve. The stability of individual RGswas evaluated using GeNorm software, a Microsoft Excel program available at https://genorm.cmgg.be/. Friedman's Two Way ANOVA and multiple comparison method were used to ana-lyze the differences between gene expression at different time points (0, 24, 48, 72, 96 and 120 hrs) of cultivation. Differences were considered statistically significant at the level of $p < 0.05$.

Results

Analysis of RNA quality

RNA purity determined as the 260 nm/280 nm absorbance ratio was 2: 1.97 for all samples. RNA integrity was assessed as the RNA Integrity Number (RIN). Ranging from 6.5 to 9.5 for allRNA samples, RIN showed good quality of RNA (with 1 being the most degraded sample and10 being the most intact). Nevertheless, RNA isolated from freshly prepared islets and islets cul-tivated for 24 hrs exhibited partial fragmentation (RIN 6.5±0.26 and 7.4±0.12, respectively). In contrast, when culturing islets for 48 hrs or more, the RIN was close to 10 (RIN 2: 9.4) what indicates nearly intact RNA. The RNA electropherograms are shown in Fig 1A. A detailed

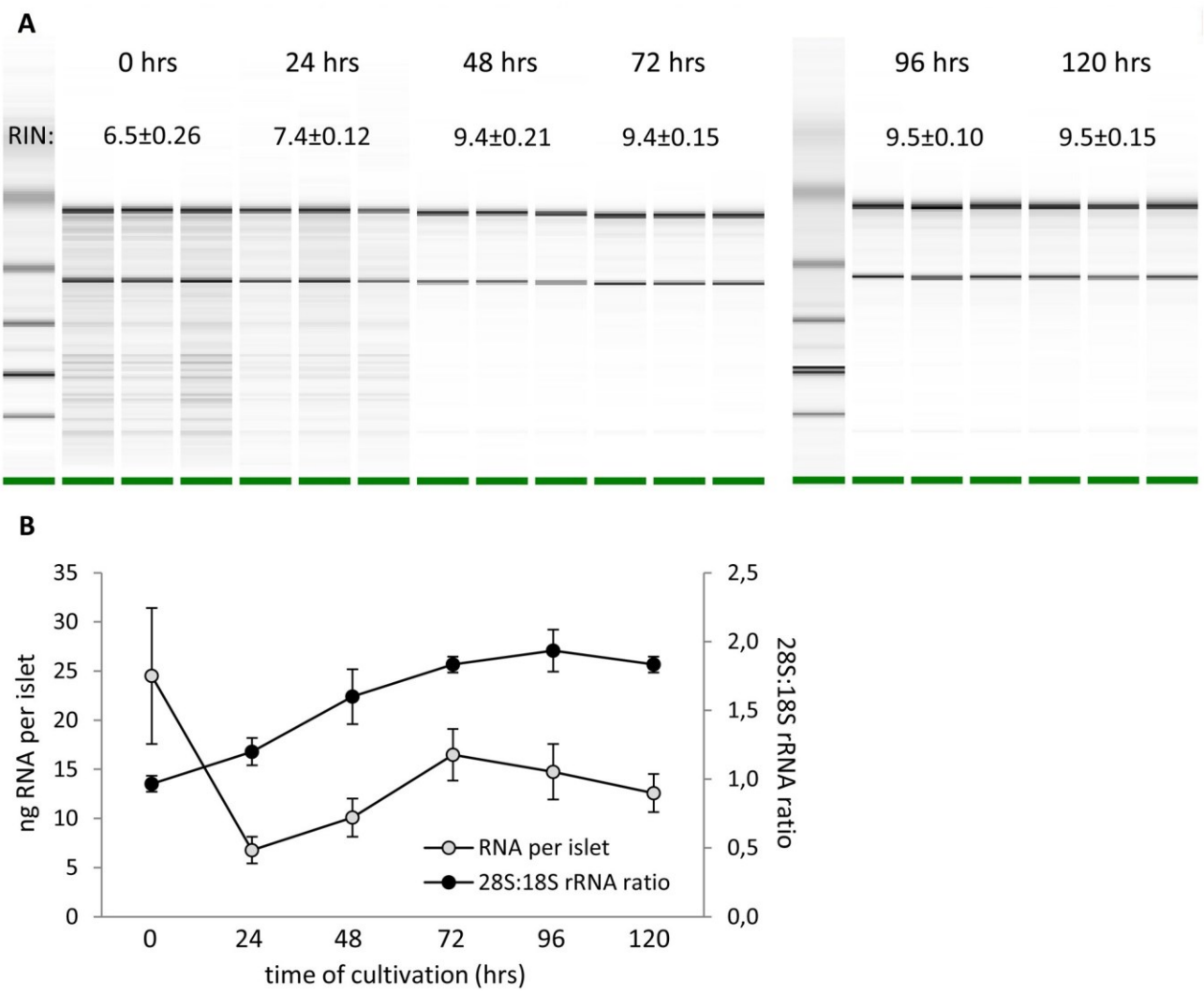

Fig 1. RNA integrity and quantity. A: Electropherograms of RNA isolated from islets at different time of cultivation. RIN = RNA integrity number. B: RNA quantity per one islet and 28S:18S rRNA ratio in the same RNA samples.

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quantification of individual electropherogram band densities is shown in S2 Fig. The 28S:18S rRNA ratio, another parameter reflecting RNA integrity, increased from 1.0 at time 0 hrs to 1.9 at 96 hrs and stabilized at 1.8 at 120 hrs of cultivation. We found the highest RNA yield per isletimmediately after isolation (T = 0 hrs) followed by significant drop during next 24 hrs and con-sequent elevation up to 72 hrs of cultivation (Fig 1B).

Metabolic characterization of islets

The metabolic state of isolated islets in various time points of cultivation was evaluated accord-ing to their capacity to respond to glucose stimulation *in vitro* (Fig 2) and by the verification ofinsulin presence by dithizone staining (Fig 3). The ability of the isolated islets to increase insu- lin secretion after glucose challenge did not significantly change throughout the 120 hrs culti-vation period. The stimulatory index (ratio of insulin secretion in basal and stimulated conditions) was highest at 6 hrs (16±2.5) and 24 hrs after isolation (19±5.3). Nevertheless,

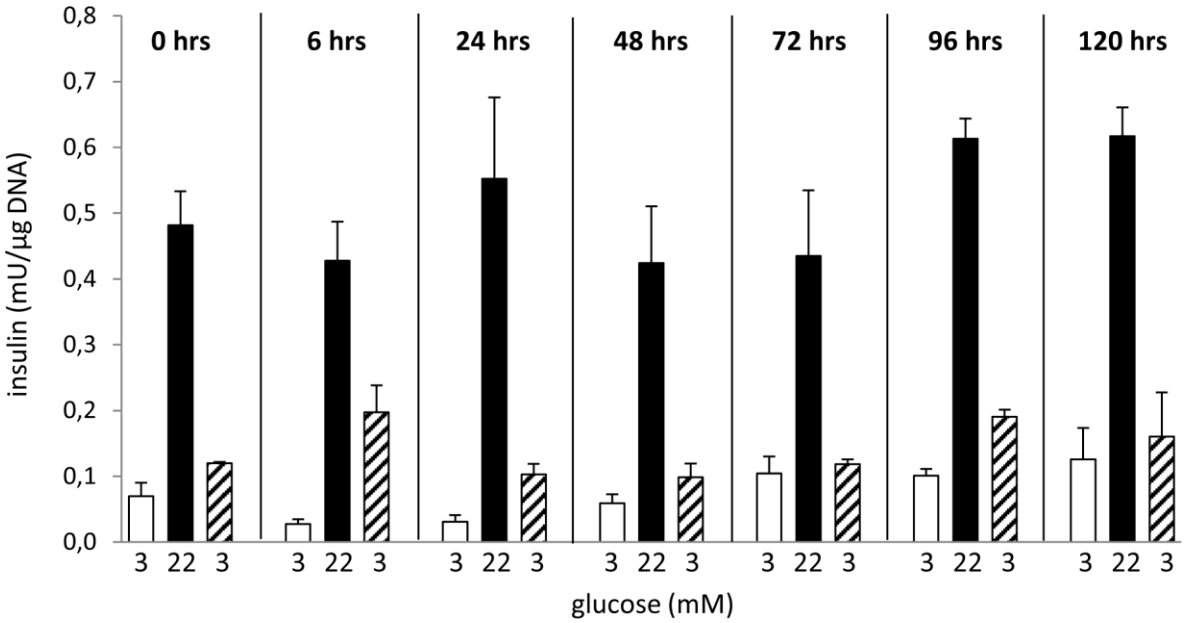

Fig 2. Effect of the length of cultivation on glucose-stimulated insulin secretion (GSIS) of pancreatic islets *in vitro*. Glucose-stimulated insulin secretion was tested at three subsequent concentrations of glucose (3 mM, 22 mM, and 3 mM again). Data are given as a mean ± SD, n = 3.

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from the absolute values (Fig 2) it is clear, that this value reflects rather low basal than elevated glucose-stimulated insulin secretion. Since 48 hrs of cultivation onwards the stimulation index was quite stable (6.1±2.0). The well-preserved metabolic flexibility of cultivated islets is demon-strated by their ability to decrease insulin secretion when transferred back to low glucose

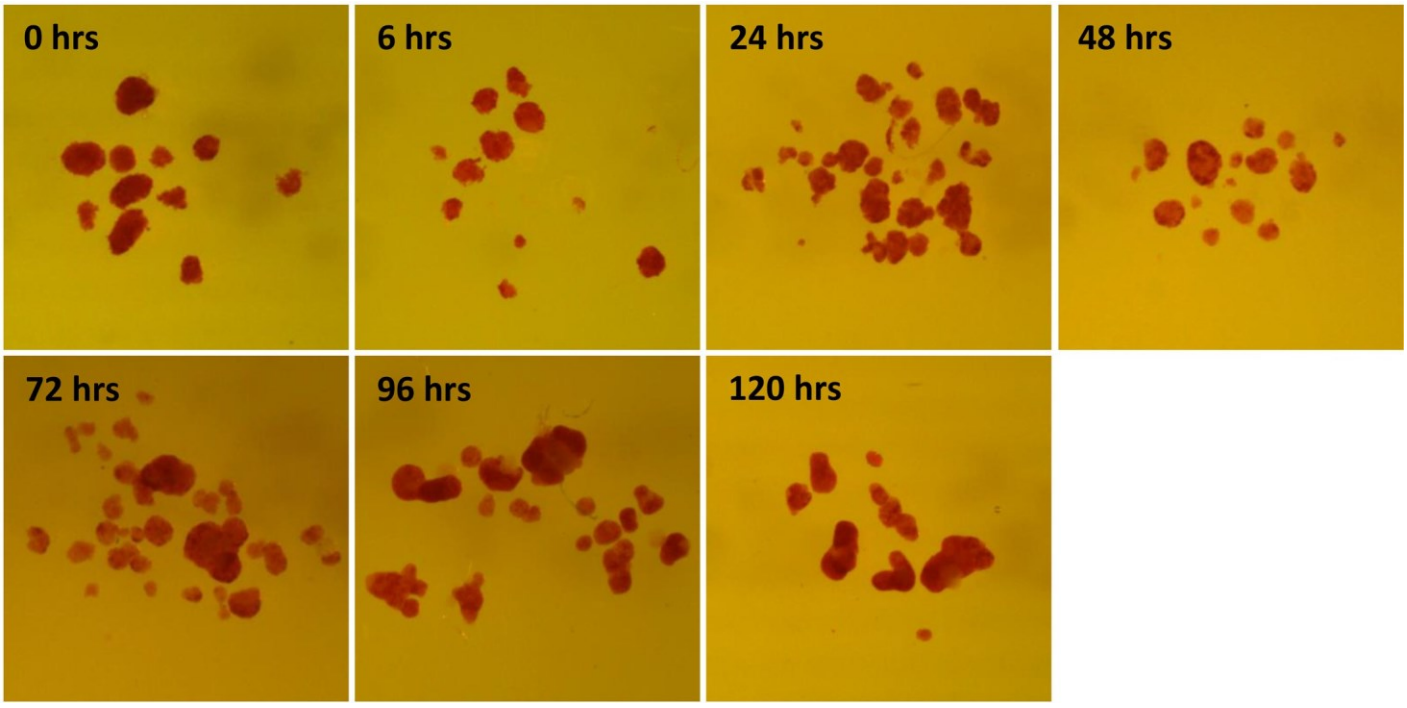

Fig 3. Dithizone staining of islets cultivated for different time periods. Magnification 15×.

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medium. During the cultivation, we did not observe any attenuation of dithizone stainingintensity what confirmed that insulin production was not compromised.

Expression profiling of candidate reference genes

The first requirement for RG is its stable expression. To examine the impact of the isolation procedure and subsequent cultivation on the candidate RGs, their expression was measured in freshly isolated islets (0 hrs) or islets cultivated *in vitro* for 24, 48, 72, 96 and 120 hrs. As shown in Fig 4, none of the genes tested in our study met the stability requirements over the whole cul-tivation period. In all cases, the specific mRNA level increased rapidly in the first 48 hrs of cul- tivation whereat it stabilized. During first 72 hrs of cultivation, it increased 15 times on average(from 5.8-fold for 18S rRNA to 43.1-fold for Pgk1) while about 90% of the increase occurred during the first 48 hrs of cultivation. In contrast, from 72 to 120 hrs of cultivation, the specificmRNA level remained relatively stable with fold change of about 1.5 on average. The only significant changes in the interval 48–120 hrs were found in the expression of 18S rRNA and Arbp gene. Furthermore, variability which was very high among samples at 0 and 24 hrs decreased significantly after 48 hrs of cultivation.

In order to learn more about the course of events during the early phase of cultivation (0–24hrs), we determined the RGs' expression every 3 hrs (Fig 5). For all genes, the expression pat- tern shared some common features. In general, the highest expression rate was observed at 3 hrs after isolation. Then the expression rate decreased and was relatively stable between 6 and15 hrs after isolation. The time interval from 18 to 24 hrs was characterized by significant vari- ability both among genes and individual time points.

The changing expression of RGs throughout the cultivation period may not necessarily dis- qualify them as normalizers for relative quantification at specific time point of cultivation, pro-vided that $\Delta Ct_{(GOI-RG)}$ is nearly constant for one time point and one particular gene of interest (GOI)/RG combination. In order to test this presumption, we calculated $\Delta Ct_{(GOI-RG)}$ for differ-ent time points of cultivation for all candidate RGs and F3 (GOI) mRNA (Fig 6). At the time after isolation and after the first 24 hrs of cultivation, $\Delta Ct_{(GOI-RG)}$ was highly variable and the coefficient of variation (CV) oscillated in hundreds of percent for all genes. In contrast, from 48 hrs onwards, expression of both GOI and RGs stabilized and $\Delta Ct_{(GOI-RG)}$ remained constantwith CV not exceeding units of percent. Taken together, these data indicate that gene expres- sion is significantly disturbed by the isolation procedure, at least at transcription level, and its stabilization occurs only after 48 hrs of cultivation.

Stability of reference genes with respect to individual stages of pancreatic islets cultivation

There are several methods used for the identification of the most stable gene combinations. The GeNorm algorithm enables the gene stability measure *M* to be calculated as the average pairwise variation (V) between one particular gene and all other candidate RGs. Genes with the lowest *M* value have the most stable expression [18]. Fig 7 shows the *M* values for individ-ual genes defined by GeNorm at 0, 24, 48,72, 96 and 120 hrs of cultivation and over the wholecultivation period (0–120 hrs). Rather surprisingly, all calculated *M* values lay well below the arbitrarily suggested cut-off value (M = 1.5) for unstable genes. On the other hand, we did notfind any set of RGs that would provide an equal rate of expression stability at all time points of cultivation. Pairwise variation analysis did not indicate the beneficial effect of including more than two RGs, as the *V* value was well below the recommended cut-off value of 0.15 (data not shown).

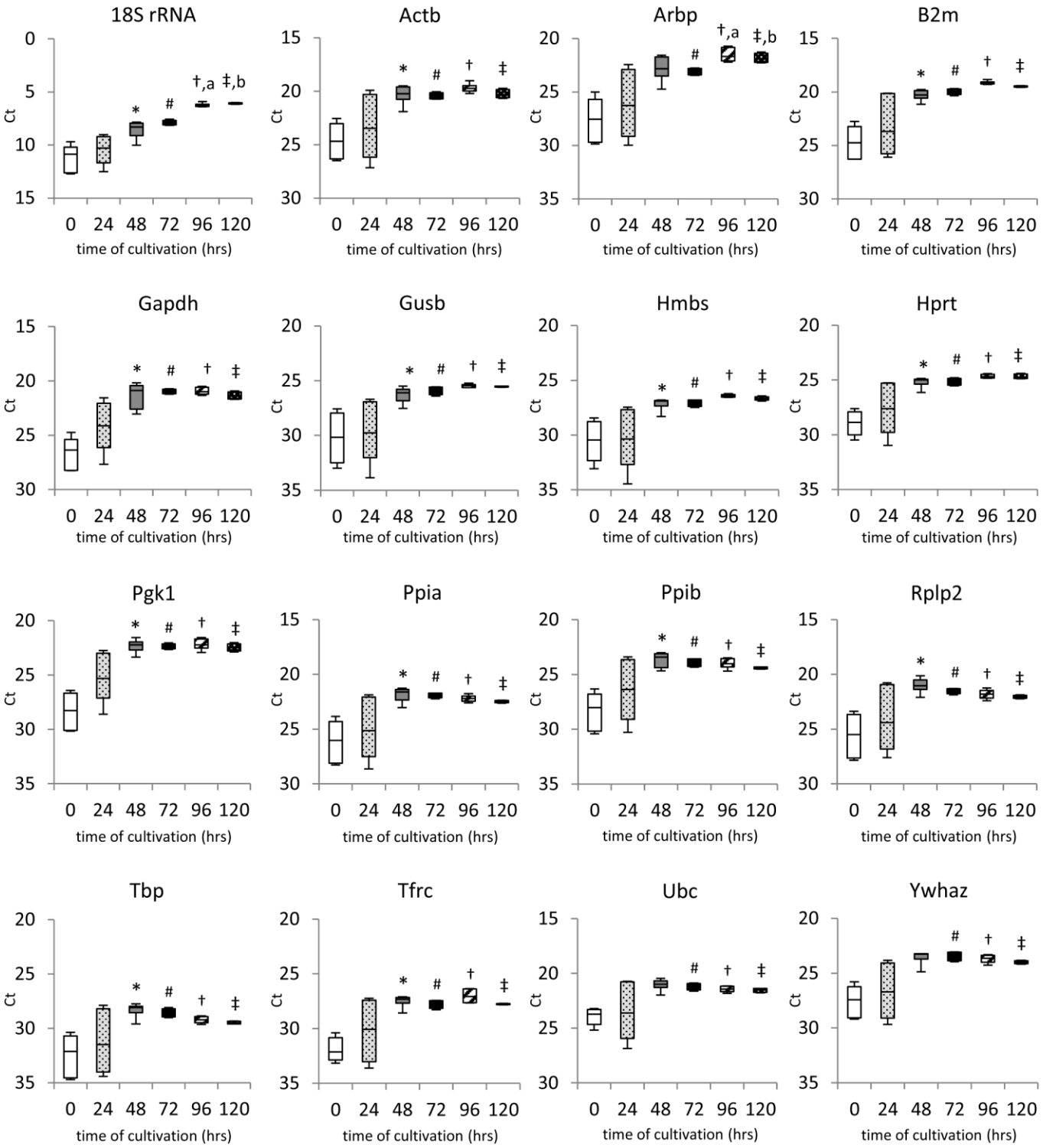

Fig 4. Expression of candidate reference genes during cultivation. Ct of individual candidate genes are shown as medians (lines), 25th percentile to the 75th percentile (boxes) and as ranges (whiskers) immediately after isolation and at 24, 48, 72, 96 and 120 hrs of cultivation. Data are based on at least 6 independent experiments. Statistical significance of the differences in Ct values was evaluated using Freidman's Two Way ANOVA and multiple comparison method; *$p < 0.05$ 48 vs. 0 hrs; $^{\#}$ $p < 0.05$ 72 vs. 0 hrs; †$p < 0.05$ 96 vs. 0 hrs; ‡ $p < 0.05$ 120 vs. 0 hrs; ^{a} $p < 0.05$ 96 vs. 48 hrs; ^{b} $p < 0.05$ 120 vs. 48 hrs.

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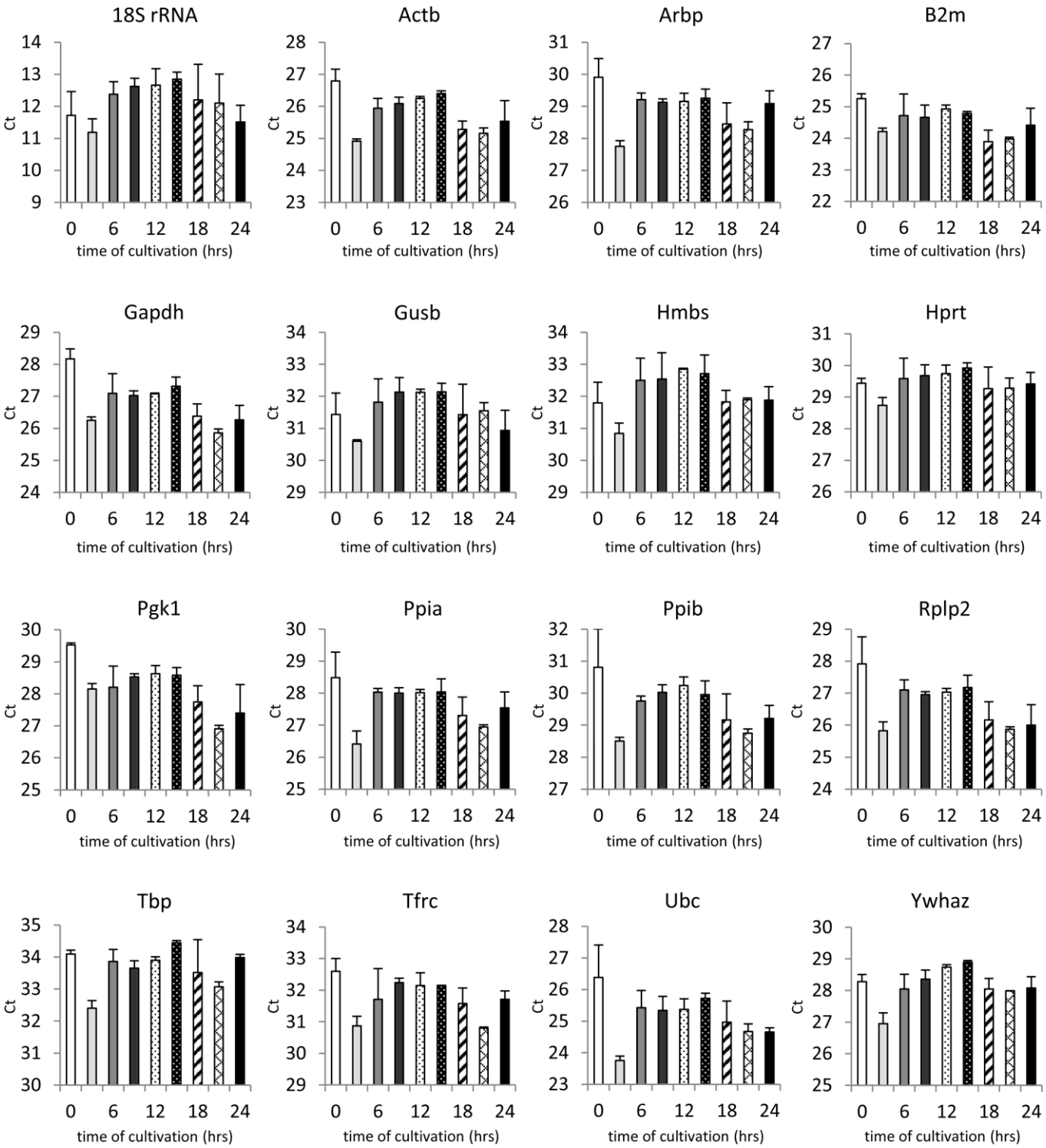

Fig 5. Expression of candidate reference genes during the first 24 hours of cultivation. Ct of individual genes are shown as mean ± S.D., n = 6. Data are based on two independent experiments.

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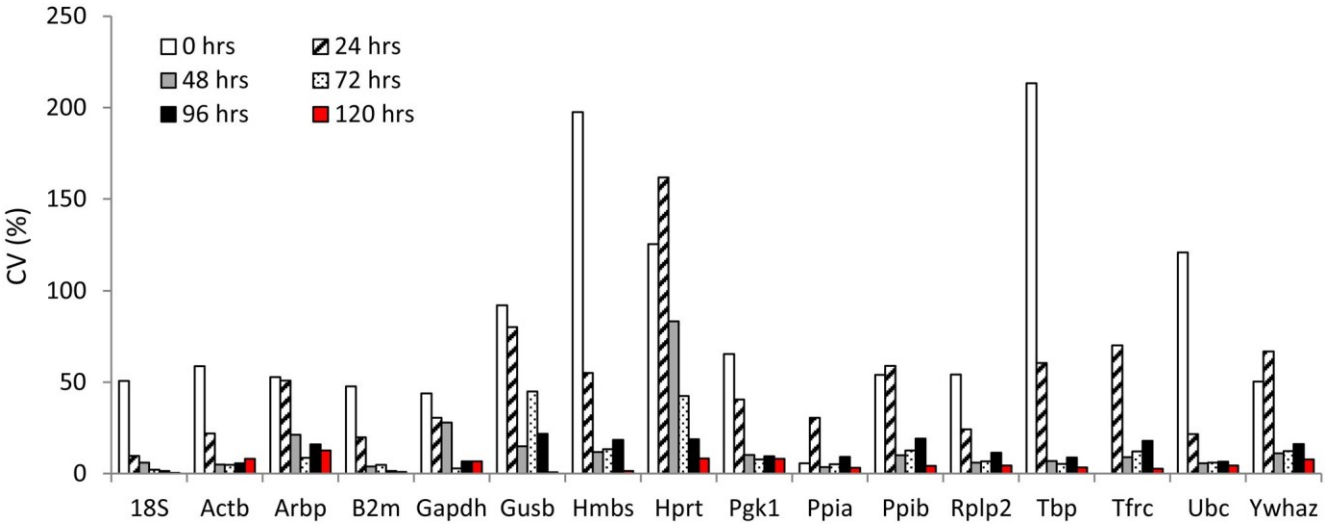

Fig 6. Coefficient of variation (%) $\Delta Ct_{(GOI-RG)}$ at different time points of cultivation. Data are based on at least 6 independent experiments.

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Another approach has been published by Gorzelniak et al. [19]. According to their guide- line, the expression of proper RG should not differ by more than half of the cycle (ΔCt S 0.5) between control and experimental samples. Employing this criterion, we evaluated the suitabil-ity of all 16 candidate RGs. In order to make the statistical evaluation possible, we converted ΔCt data to "fold changes" using the $2^{-\Delta Ct}$ method. In this case, ΔCt of +0.5 and -0.5 are equiva-lent to 0.7 and 1.4-fold changes in relative gene expression, respectively [20]. Because gene expression in our experiment seemed to stabilize at 48 hrs, we chose average expression of therespective candidate RGs at 48 hrs as a calibrator. None of the tested genes met the ±0.5 ΔCt rule in freshly isolated islets or at 24 hrs after isolation (Fig 8A, 8B and 8C). When considering only the period from 48 to 120 hrs of cultivation, eight of the genes (18S, Actb, Arbp, B2m, Gusb, Ppib, Tbp, Tfrc) (Fig 8A) were out of the limit in at least one of cultivation times evalu-ated. The average expression of six genes (Gapdh, Hmbs, Pgk1, Ppia, Rplp2, Ywhaz) met the ±0.5 ΔCt rule during the interval 48–120 hrs but due to the variance their expression in some individual samples exceeded 0.7 or 1.4-fold change borders (Fig 8B). The only two genes thatabsolutely complied with the ±0.5 ΔCt rule were Hprt and Ubc (Fig 8C).

Comparison of relative vs. absolute quantification of gene expression in isolated pancreatic islets

In order to test the plausibility of relative quantification of gene expression at different time points during pancreatic islet cultivation, we employed the $2^{-\Delta\Delta Ct}$ method when the expressionat 48 hrs was set as calibrator and calculated the expression of three genes appointed as "GOIs"using three RG combination: 1) most stable pair identified by GeNorm at 24 hrs; 2) most stablepair identified by GeNorm over the whole cultivation period (0–120 hrs) and 3) RG pair identi-fied by the ±0.5 ΔCt rule. We compared these results with data obtained by the absolute quanti-fication method which determined the respective mRNA concentration using a calibration curve constructed for the specific transcript (Fig 9A, 9B and 9C). We chose the following genesof interest: Gapdh, one of the most widely used RGs; Ppia, identified as one of the most stable genes in our experimental setting; and F3 (tissue factor), which is referred to increase its expression during the *in vitro* cultivation [21].

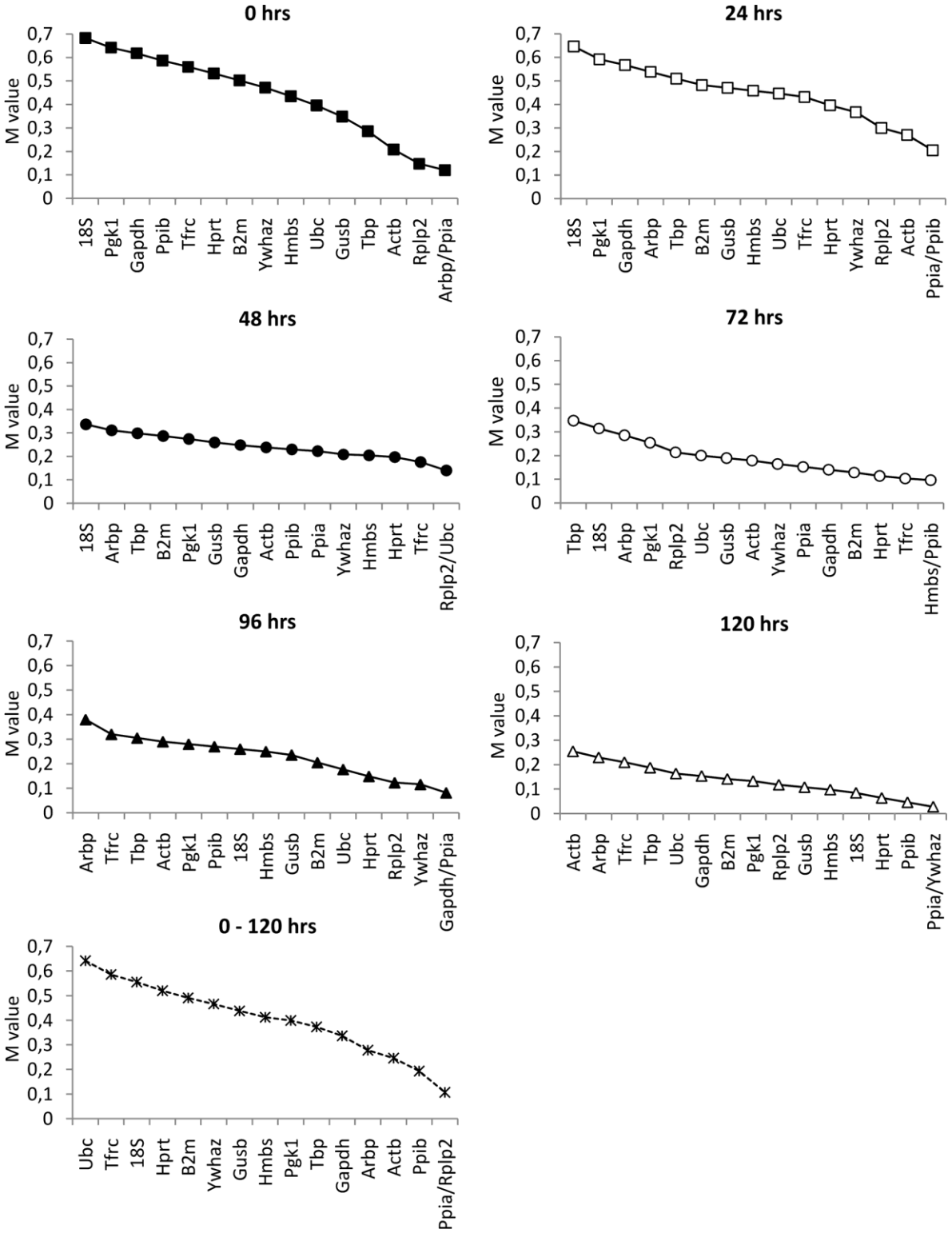

Fig 7. Expression stability values of candidate RGs at different phases of cultivation determined by GeNorm. The *M* value represents an average stability measure of each possible combination of a particular RG with all other genes in the multiplex. The lower the *M* value of a given gene, the more consistent its expression relative to other genes in the multiplex.

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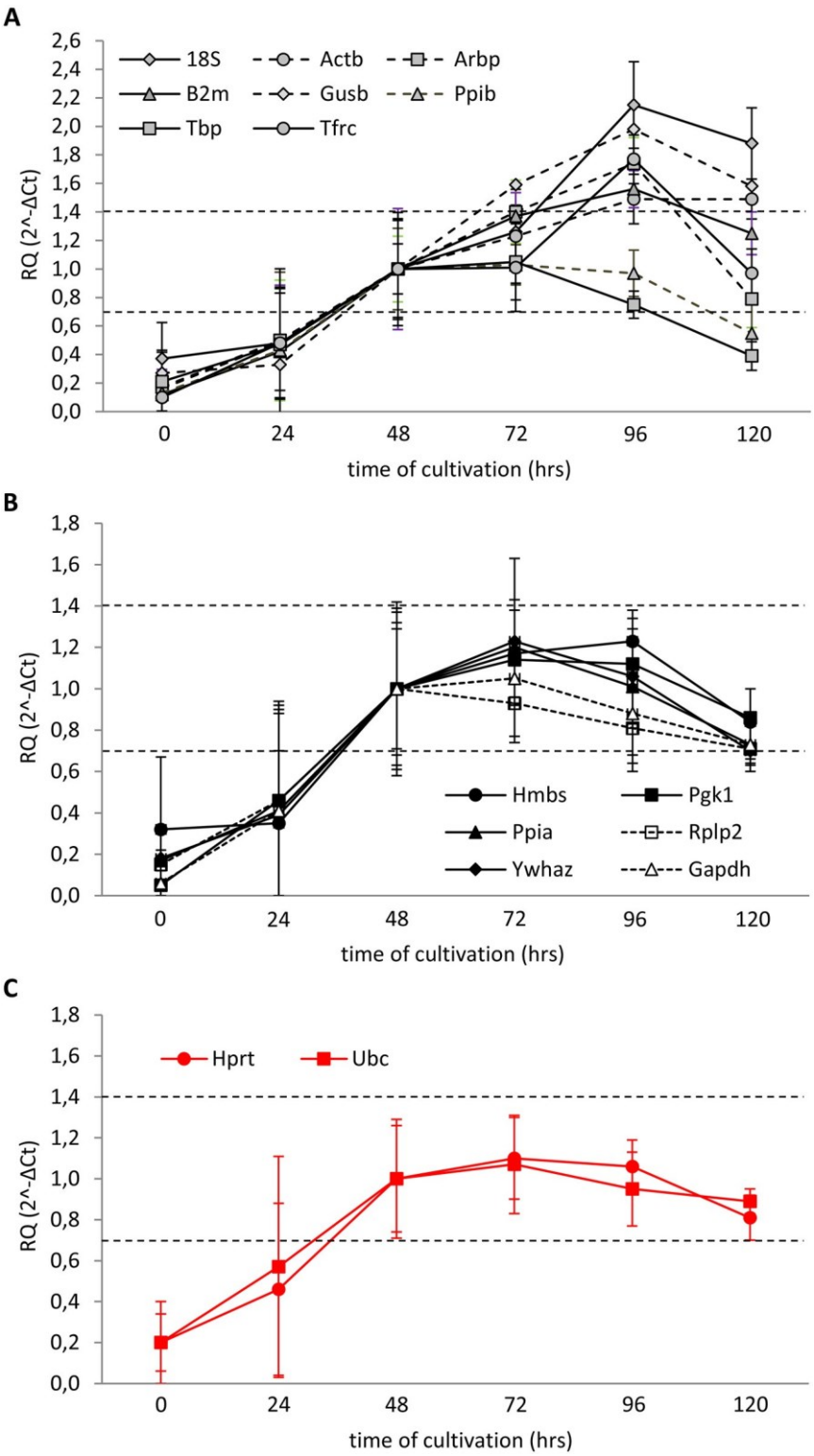

Fig 8. Expression stability of candidate RGs at different phases of cultivation evaluated according to the ±0.5 ΔCt rule. Data are expressed as means ± SD, n = 6. Dashed lines indicate the interval of 0.7-1.4-fold change compared with the expression at 48 hrs.

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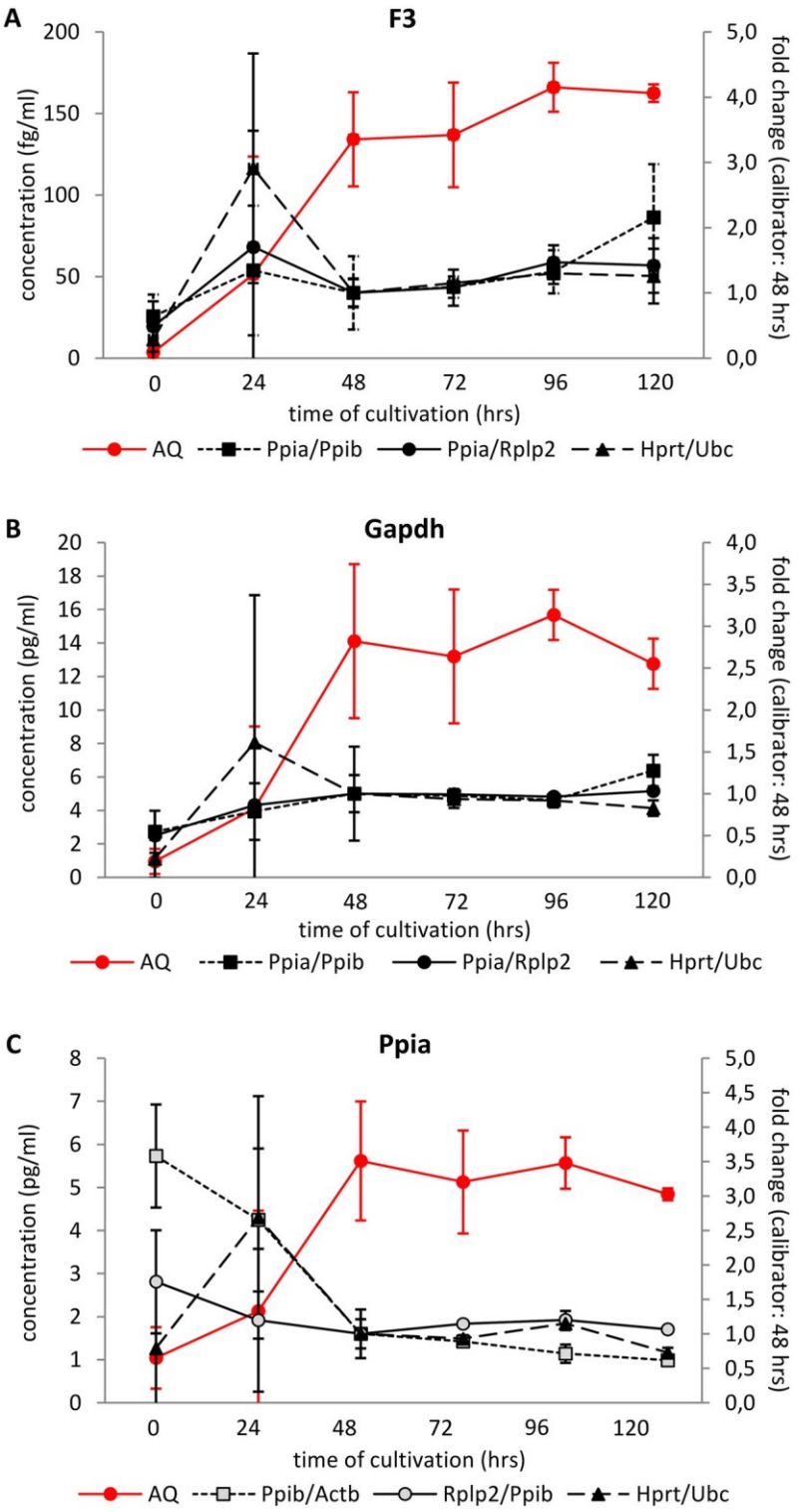

Fig 9. Relative and absolute quantification of F3, Gapdh and Ppia in isolated pancreatic islets during *in vitro* cultivation. Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method related to time 48 hrs; absolute expression was determined using a calibration curve constructed for each specific transcript. RG pairs were chosen either by GeNorm, calculated for expression at 24 hrs only (Ppia/Ppib), or for the whole interval 0–120 hrs (Rplp2/Ppia) or by the ±0.5 ΔCt rule (Hprt/Ubc). When Ppia was evaluated as GOI, we used the next most stable reference gene identified by GeNorm (24 hrs: Ppib/Actb; 0–120 hrs: Rplp2/Ppib).

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According to the absolute quantification method, we observed a similar pattern of expres-sion for all three GOIs—their expression was very low at the time of isolation and then increased up to 48 hrs of cultivation after which it remained relatively stable. In contrast to this, the results of relative quantification failed to reflect the changes in respective mRNA con-centrations determined using the absolute quantification method during the first 48 hrs for allthree GOIs irrespective to the RG pair chosen. In the interval 48–120 hrs, the expression of GOIs determined by relative expression generally paralleled their expression assessed by abso-lute quantification. Both methods identified enormous variance at 24 hrs of cultivation.

These data confirm our presumption that due to the unstable expression of most of the genes during the initial phase of *in vitro* cultivation, relative quantification of mRNA expres-sion is not a suitable method as it does not reflect the real mRNA concentrations during the first 48 hrs of cultivation.

Discussion

Our study focused on the identification and validation of a suitable set of RGs for use in gene expression normalization during short-term cultivation of isolated rat pancreatic islets. We found that none of the 16 candidate RGs met the stability criteria (ΔCt S ±0.5) throughout the whole 120 hrs cultivation period. Our main findings can be summarized as follows: first, imme-diately after isolation, the specific mRNAs content was many times lower in comparison with all subsequent phases of cultivation and RNA exhibited considerable degradation; second, dur-ing the recovery phase which lasted for subsequent 48 hrs, transcription of all tested genes increased but it was extremely variable among samples which were supposed to be homoge- nous; third, from 48 hrs of cultivation onwards, RNA integrity increased and expression levels of all tested genes stabilized with minimal variability among samples; finally, none of the candi-date RGs met the acceptable fluctuation criteria (ΔCt S ±0.5) during the first 48 hrs of cultiva- tion. Taken together, our data suggest that normalization during the first stages of pancreatic islet cultivation is problematic and raise questions as to the suitability of gene expression stud- ies and particularly of the relative quantification method under these conditions.

RT-qPCR is a widely-used method for quantitative determination of gene expression in bio-logical samples; however, it is also quite prone to a number of imprecision and technical errors.These problems could be overcome by normalization, i.e. including an invariant endogenous control in the assay to correct both sample-to-sample variations in RT-qPCR efficiency and errors in sample quantification. However, it is essential that expression of the selected endoge- nous control (i.e. RG) is stable and not affected by the experimental conditions used in the study under investigation [1,4].

In our experiment, we found that immediately after pancreatic islet isolation, expression ofall tested candidate RGs was significantly down-regulated when compared to that at 48 hrs of cultivation (5- to 40-fold, 13,5-fold in average). This is probably due to the ischemic, mechani- cal, osmotic, and oxidative stress to which pancreatic islets are exposed during the isolation procedure [22]. As previously published, during the process of isolation, islet cells undergo pro-found changes in structure and function resulting in beta-cell apoptosis [23], whether as a con-sequence of hypoxia, disruption of the native islet cell microenvironment or lack of growth factors [23–27]. Furthermore, the isolation procedure potently recruits some of stress signalingpathways; however, culturing of islets for 48 hrs after isolation allows for activated pathways to return to background levels [28].

In order to verify the quality of RNA isolated from islets harvested at different time pointsof cultivation, we measured integrity and possible degradation of RNA as it may also influencethe results of RT-qPCR analysis. According to RIN and the corresponding electropherograms

(Fig 1A and S2 Fig), RNA obtained from freshly isolated islets and from islets cultivated for 24 hrs exhibits partial fragmentation (RIN 6.5±0.26 and 7.4±0.12, respectively) while the RNA from islets cultivated for 48 hrs or more is nearly intact (RIN 2: 9.4). 28S:18S rRNA ratio, another parameter reflecting the degree of RNA degradation, was low in freshly isolated islets (1.0) but then rose up and got stabilized (1.9 and 1.8) after 96 and 120 hrs of cultivation, respec-tively. Finally, the amount of RNA obtained from one islet was highest in freshly isolated islets, then it dropped rapidly, reached the minimal value after 24 hrs and then increased slowly again(Fig 1B). All these observations could be explained by launching of degradation processes through the stress to which islets are exposed during the isolation procedure [28]. These mech-anisms probably lead to general destruction of RNA resulting in rapid decrease in RNA contentduring the first 24 hrs after isolation. In tissue culture, as the degradation and stress signaling pathways are not stimulated anymore [28], the RNA content start to rise and the islet conditionimproves. After 48 hrs of cultivation, there is no evidence of RNA degradation (Fig 1). Never-theless, the initial RNA content per one islet is not reached again, probably due to natural cell death, eventually also due to central necrosis of some islets during cultivation. For these rea- sons, along with the time of cultivation, the number of islet cells naturally decreases which means that the RNA content per one islet drops as well. Interestingly, the main metabolic func-tion of the islets, the ability to respond to glucose stimulation by the increased insulin secretion,was not compromised at any time point of cultivation. The intactness and the presence of insu-lin in the islets throughout the cultivation were evidenced by dithizone staining. Taken together, our data indicate that isolation stress affects particularly the RNA stability and/or synthesis while the main metabolic characteristics are unaffected.

According to our observations, during the first 48 hrs of cultivation, significant fluctuationsin gene expression occurred, while from 48 hrs onwards, substantial stabilization took place. Particularly, 24 hrs after islet isolation, gene expression of all tested genes was extremely vari-able among the samples which were supposed to be homogenous. Also in the time period 0–24hrs, the expression of all genes varied significantly both among times and genes. In contrast, after 48 hrs of cultivation, Ct values stabilized with minimal variability among samples, for most of the genes. This observation questions the biological relevance of gene expression stud-ies during the early phase (0–48 hrs) of islet cultivation *in vitro*.

Several approaches have been developed for selection of a suitable internal control. The "empirical" approach is based on selecting genes with robust expression, i.e. Gapdh, B2m, or 18S rRNA, which have been successfully used in non- or semiquantitative methods, such as Northern blot where qualitative changes are measured [29]. Even though there is a wealth ofevidence indicating that these genes are significantly affected by different conditions [2,7– 12,30], they are still frequently used in gene expression studies carried out on isolated pancre-atic islets [12,31]. In our experiment, some of these genes (e.g. 18S rRNA) were found to be quite unstable (Figs 4, 7 and 8).

Another approach is based on the precise evaluation of RG stability under particular experi-mental conditions. According to Gorzelniak [19], differences in ΔC_T between "experimental" and "control" sample for particular RG < 0.5 are caused by technical variance of the method and are likely to be reflected by the gene of interest and RG in the same manner. In contrast, ΔC_T values >1.0 reflect changes in gene expression levels and indicate that candidate RG expression is influenced by experimental conditions. In our study, ΔCt ranges from 2.3 to 5.3 during the first 48 hrs after isolation. Therefore, the use of any of 16 candidate RGs as normal- izers for relative quantification of gene expression during the first 48 hrs of cultivation is inap- propriate and would lead to erroneous results. However, after 48 hrs of cultivation, expression of eight of the potential RGs (Gapdh, Hmbs, Hprt, Pgk1, Ppia, Rplp2, Ubc, Ywhaz) stabilized with the ±0.5 ΔCt range. Although the expression level of some genes moved slightly up or

down from 72 hrs onwards, the variability of expression among samples in one groupdecreased significantly (Figs 4 and 6).

The GeNorm method [18] was developed for determination of gene expression stability on the basis of non-normalized expression levels. The GeNorm algorithm allows gene-stability measure *M* to be calculated as the average pairwise variation (*V*) between the particular gene and all other candidate RGs. In contradiction to our findings, all calculated *M* values providedby GeNorm lay well below the arbitrarily suggested cut-off value (*M* = 1.5; with a lower valueindicating increased gene stability), which means that all combinations of two tested genes should be stable enough to be used as internal controls. Nevertheless, as shown in Figs 4 and 8,expression of all candidate RGs significantly increased during the first 48 hrs of cultivation.
This discrepancy could be explained by the mechanism used for GeNorm calculation, which evaluates the stability of each combination of two genes remaining in a multiplex after one-by- one exclusion according to a descending *M* value until only two genes with the most stable expression ratio remain. Due to the GeNorm's pairwise comparison these cannot be further differentiated. Because the expression of all genes during the 48 hrs after isolation followed a similar pattern, the ratio between every two compared genes appeared to be stable. This may also explain why there was no need to include more than two RGs according to GeNorm's pair-wise variation analysis, since the calculated *V* value fell well below the recommended cut-off value of 0.15 (data not shown).

Finally, the limited suitability of relative quantification in isolated pancreatic islets during the first 48 hrs of cultivation is illustrated if we compare the results of relative and absolute quantification of the three genes: F3, Gapdh, and Ppia (Fig 9). F3 gene encodes the protein of tissue factor which plays a key role in tissue engraftment when performing pancreatic islet transplantation. Tissue factor triggers the Instant Blood Mediated Inflammatory Reaction (known as IBMIR) immediately after pancreatic islet infusion into the portal vein blood, thus influencing the ratio of destroyed/engrafted islets. The increasing expression of F3 gene during tissue culture preservation of islets before transplantation was repeatedly reported. Gapdh genewas chosen as one of the most "popular" housekeeping genes used as normalizers in gene expression analysis. Ppia gene was selected as it seems to be one of the most stable genes in our experimental setting (Fig 7). Absolute quantification showed a similar expression pattern for all three genes. The gene copy number per sample was very low immediately after isolation, then increased over the next 48 hrs and remained relatively stable between 48 hrs and 120 hrs.In contrast to this, the results of relative quantification failed to reflect the changes in respectivemRNA concentrations determined using the absolute quantification method during the first 48hrs for all three GOIs irrespective to the RG pair chosen. In the interval 48–120 hrs, the expres-sion of GOIs determined by relative quantification generally paralleled the expression assessed by absolute quantification. Better results were obtained when using RG pairs identified accord-ing to their expression during the whole cultivation period both by GeNorm or ±0.5 ΔCt rule than by RG pair chosen according to the expression at one particular time point, i.e. at 24 hrs.

In conclusion, we provide here evidence that during the first 48 hrs of isolated pancreatic islet cultivation, expression of commonly used RGs is unstable apparently due to the isolationstress which probably leads to nonspecific RNA destruction and activation of RNA degradationpathways. During this period, data obtained using relative quantification do not reflect actual changes in specific mRNA concentrations and related calculations can lead to misinterpreta- tion of study findings. Therefore, we suggest that other methods rather than commonly used relative quantification should be used to determine gene expression in freshly isolated pancre- atic islets or islets should be stabilized in tissue culture 48 hrs before gene expression studies.
From 48 hrs onwards, relative quantification method can provide reliable information in

isolated pancreatic islets. Our findings highlight the importance of RG validation step for everyparticular experimental setting.

Supporting Information

S1 Fig. Representative samples of dithizone stained islets at 0, 24, and 48 hrs after isolation.
Magnification 15×.
(PDF)

S2 Fig. Electropherograms of RNA samples isolated from islets at different time points ofcultivation.
(PDF)

S1 Table. Official symbol, ID, full name, function and location of 16 candidate referencegenes and the F3 gene. Available at http://www.ncbi.nlm.nih.gov/gene/.
(PDF)

Author Contributions

Conceived and designed the experiments: LK MC JK. Performed the experiments: LK EF IL TK. Analyzed the data: LK MC IT. Contributed reagents/materials/analysis tools: LK JK FS.Wrote the paper: LK MC JK FS.

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RESEARCH PAPER

A novel model for *in vivo* quantification of immediate liver perfusionimpairment after pancreatic islet transplantation

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ABSTRACT

Instant Blood-Mediated Inflammatory Reaction (IBMIR) is a major cause of graft loss during pancreatic islet transplantation, leading to a low efficiency of this treatment method and significantly limiting its broader clinical use. Within the procedure, transplanted islets obstruct intrahepatic portal vein branches and consequently restrict blood supply of downstream lying liver tissue, resulting typically in ischemic necrosis. The extent of ischemic lesions is influenced by mechanical obstruction and inflammation, as well as subsequent recanalization and regen- eration capacity of recipient liver tissue. Monitoring of immediate liver perfusion impairment, which is directly related to the intensity of post-transplant inflammation and thrombosis (IBMIR), is essential for improving therapeutic and preventive strategies to improve overall islet graft survival. In this study, we present a new experimental model enabling direct quantification of liver perfusion impairment after pancreatic islet transplantation using ligation of hepatic arteries followed by contrast-enhanced magnetic resonance imaging (MRI). The ligation of hepatic arteries prevents the contrast agent from circumventing the portal vein obstruction and enables to discriminate between well-perfused and non-perfused liver tissue. Here we demonstrate that the extent of liver ischemia reliably reflects the number of transplanted islets. This model represents a useful tool for *in vivo* monitoring of biological effect of IBMIR-alleviating interventions as well as other experiments related to liver ischemia. This technical paper introduces a novel technique and

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1. Introduction

After the introduction of the Edmonton protocol in 2000, transplantation of isolated pancreatic islets has become a clinically applicable alternative to solid pancreas transplantation for the therapy of type-1 diabetic patients, especially those prone to hypoglycemia unawareness syndrome.[1,2] In most cases, transplantation is performed as an infusion of islet suspension into the hepatic portal vein, thus allowing the islets to settle spontaneously in its peripheral branches. Transplanted islets cause mechanical obstruction and significantly reduce blood perfusion of downstream-lying liver tissue. The simple mechanical obstruction is intensified by the Instant Blood-Mediated Inflammatory Reaction (IBMIR), which is characterized by platelet aggregation, activation of clotting cascade and complement system, infiltration of leukocytes into the islets, and formation of thrombi as a consequence of direct contact of islet cells with recipient blood. IBMIR starts within minutes and culminates about 120 min after islet transplantation.[3,4] Focal discolora- tions, which develop in suboptimally/insufficiently perfused liver tissue, are clearly visible immediately after islet transplantation with subsequent progression into focal necrosis of liver cells in order of hours. The consequent inflammation and thrombosis lead to a significant loss of transplanted islets early after infusion. It is estimated that 50–70% of transplanted islets are subject to destruction, i.e., less than half of the islets successfully engraft.[3-7] Taken together, IBMIR contributes significantly to the fact

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that islet grafts isolated from more than one donor are required to achieve insulin independence of a diabetic patient in clinical practice.[4,7-9]

Several intervention strategies improving islet engraftment by inhibition of coagulation and/or inflammation have been reported,[8-14] however, a technique for quantification of ischemic liver tissue is still missing despite its critical need for the evaluation of efficiency of the diverse therapeutic interventions. Until now, IBMIR monitoring was based merely on the measurement of the level of thrombin-antithrombin complex after pancreatic islet transplantation or *in vitro* using the blood loop system.[8-11,13] To our best knowledge, only one paper focused on morphological quantification of necrotic liver tissue after islet transplantation was published.[15] The reported study was performed using a mouse animal model, a high number of transplanted islets (≳30 islets/g of body weight), and subsequent *ex vivo* examination of recipient liver using magnetic resonance imaging (MRI) with 11.7T scanner lasting for 80 min. Necrosis detected in sacrificed animals is characteristic for the late state of liver damage, but the obtained data does not enable the quantification of the actually non-perfused but still vital tissue. Therefore, this study proved the ability of MRI to examine the late liver impairment after islet transplantation in principle; nevertheless, the employed methodology cannot be used for *in vivo* monitoring of interventions aimed at modulating IBMIR.

The main aim of this study was to prepare a novel de-arterialized liver model for visualization of liver ischemia after pancreatic islet transplantation, which would be applicable for *in vivo* evaluation of efficiency of different experimental approaches focused on inhibition of IBMIR, and to test the sensitivity of this novel method using transplantation of islet grafts of different sizes (increasing number of islets).

2. Results

In our study, we have introduced a modification of the reported model to a more sensitive variant, which enables the comparison of different intervention approaches *in vivo* (heparinization and other coating of islets, islet preconditioning by RNA interference, preculturing of islets with different substances or cells, etc.). In order to improve the discrimination threshold between hypo-perfused and control liver tissue, we injected a contrast agent into the lateral tail vein of islet recipients at the start of the MRI examination 2 h after trans- plantation, i.e., at the time of presumed IBMIR culmination. The contrast agent increases the differ- ence in signal intensity of perfused and non- perfused liver tissue, thus increasing the sensitivity of MRI. In addition, we have ligated hepatic arteries prior to islet transplantation. Without this interven- tion, hepatic arteries provide a way for the contrast agent to bypass the obstruction in portal vein blood flow. Previously, it was repeatedly reported that in case of liver transplantation in rodents, arterial reconstruction is better but not necessary for a long-term graft function and animal survival.[16,17] In contrary to the study described above, we have used a significantly lower number of islets (0, 4, −4 islets per gram), and a less powerful MRI scanner (4.7T vs 11.7T).

2.1. Macroscopic visualization of intraportal thrombosis

Immediately after pancreatic islet transplantation into the rat liver, ischemic areas appear which are clearly visible in a macroscopic view (Figure 1). Inside the liver, a large thrombus can be found filling the branches of the portal vein tree (Figure 2). Two days after transplantation, non-perfused liver areas

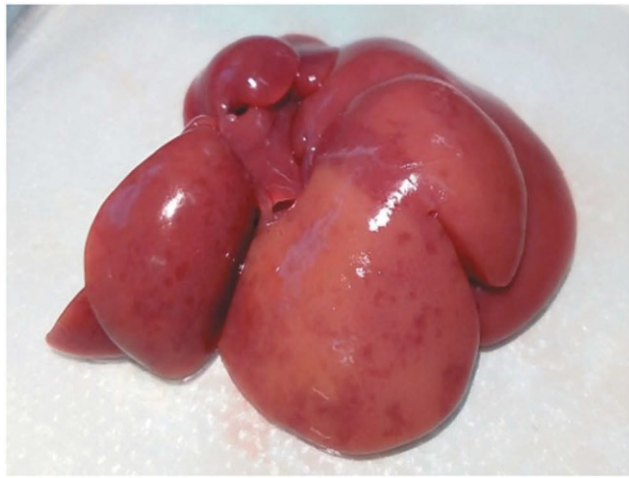

Figure 1. Ischemic areas in rat liver 2 h after transplantation of 1000 syngeneic pancreatic islets without ligation of hepatic arteries. Dark brown/violet areas represent the non-perfused liver tissue.

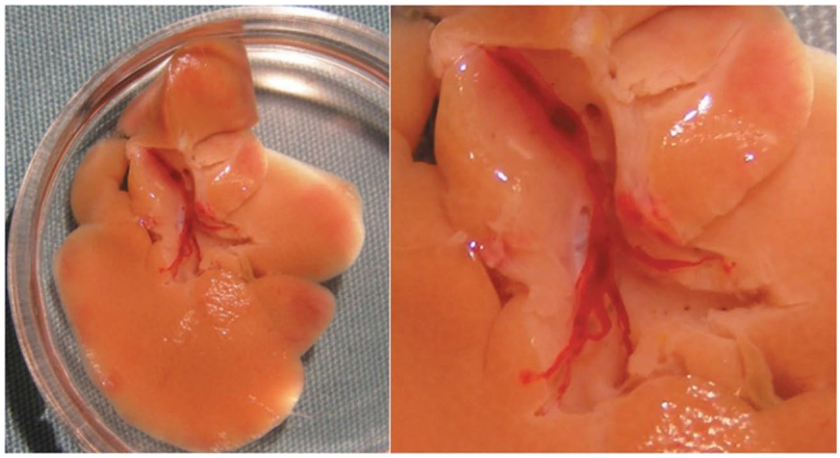

Figure 2. Large thrombus filling the branches of portal vein inside of an ischemic rat liver 2 h after transplantation of 1000 syngeneic pancreatic islets.

turn into necrotic lesions. In 28 d, lesions are completely healed (data not shown).

2.2. Intravital staining with patent blue dye

Two hours after transplantation of 100, 500 and 1000 pancreatic islet with ligation of hepatic arteries, the areas of non-perfused liver tissue were demonstratively visualized by injection of patent blue intravital contrast dye (Figure 3A–C). In case of 100 islets transplantation, ischemia was macroscopically almost indeterminable, whereas transplantation of 500 and 1000 islets led to a formation of extensive ischemic areas. Transplantation of islets without ligation of hepatic arteries caused the obstruction of several portal vein branches detectable only as temporarily delayed distribution of blue dye into these areas. Within 2 min, the hepatic arterial

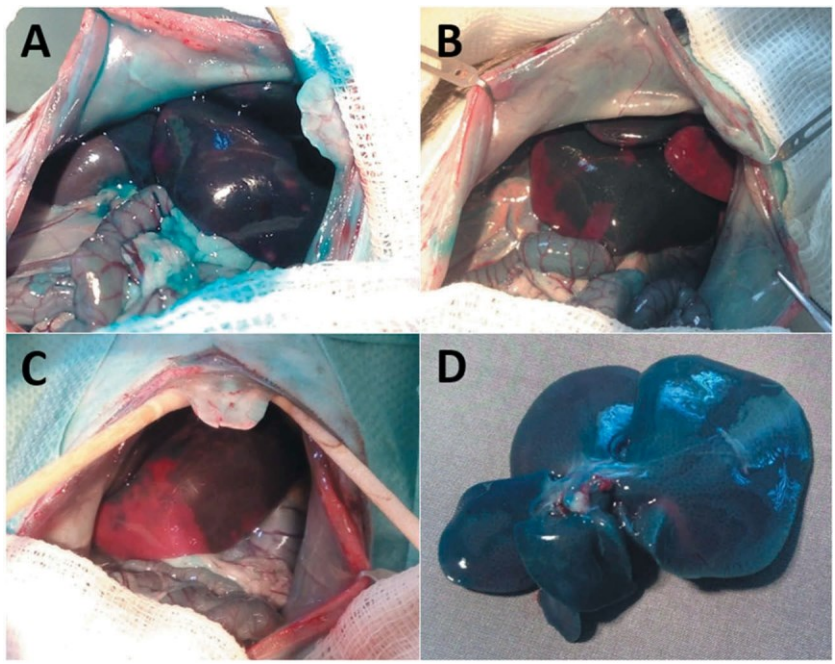

Figure 3. Non-perfused areas of liver tissue 2 h after transplantation of 100 (A), 500 (B) and 1000 (C) syngeneic pancreatic islets with ligation and excised liver after transplantation of 1000 syngeneic pancreatic islets without ligation of hepatic arteries (D) 2 min after application of patent blue intravital contrast dye. Without ligation, the contrast dye perfuses quickly into the temporally ischemic areas through the arterial circulation.

bloodstream bypassed the obstruction and spread out the contrast dye over the whole liver volume (Figure 3D).

2.3. Magnetic resonance imaging and quantification of ischemic tissue volume

The mean total volume of recipient liver measured using MRI in combination with a specific contrast agent was 9.50 ± 1.04 ml. The volume of ischemic tissue varied from 0.55 ± 0.01 ml in Group A, to 2.09 ± 0.11 ml in Group B, and 2.88 ± 0.01 ml in Group C, yielding in the proportion of ischemic tissue raising from 6.3% to 20.7%, and 28.1%, respectively, of the total liver volume (Figure 4).

2.4. Serum examination

Figure 5 shows that ligation of hepatic arteries does not have any detrimental effect on rat liver cells. A temporary increase of liver enzymes activity in blood indicating the impairment of hepatocytes was detected early after islet transplantation (day +2) and was normalized until day 7 after surgery. The evaluated biochemical parameters remained in physiological range until the end of study.[18]

3. Discussion

During the last two decades, extensive research was conducted focusing on the improvement of pancreatic islets engraftment in order to increase the efficiency of pancreatic islet transplantation as a therapeutic method. A large number of studies focused on alleviating IBMIR was published[8-14]; however, no method allowing the direct assessment of individual intervention efficiency *in vivo* has been available so far. Indirect laboratory parameters were reported indicating but not quantifying liver impairment caused by islet transplantation such as temporary increase of liver enzymes activity or presence of coagulation markers in blood.[8-11,13] Several papers also describe the decrease of islet number detected by MRI or positron-emission tomography during early posttransplant period,[5,6,19,20] but the possibility of measuring the liver damage directly was missing. The only work reporting the range of posttransplant ischemic necrosis was focused on the late, fully matured consequences of portal vein microthrombosis but not on the acute post-transplant disturbances of blood flow.[15] These conditions are not commutable as the ultimate extent of the liver necrosis is, in addition to interruption of blood supply, influenced also by other factors including the timingof examination, the activity of plasminogen system, the individual resistance to ischemia, etc. The direct

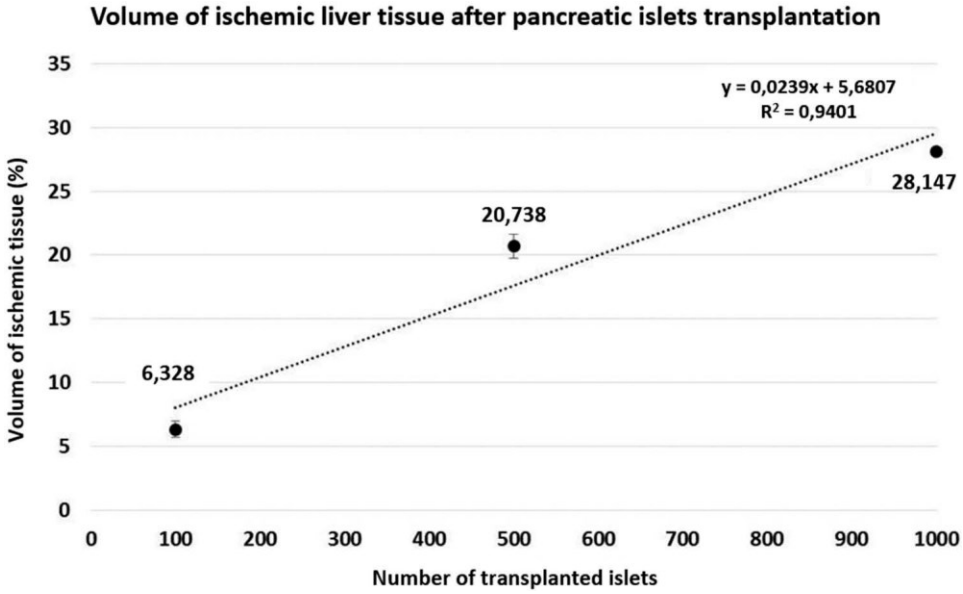

Figure 4. Calibration curve establishing a direct correlation between the number of transplanted islets and volume of ischemic liver tissue 2 h after transplantation of 100 (Group A), 500 (Group B) and 1000 (Group C) pancreatic islet into the portal vein of healthy rats (n = 6 in each group) with hepatic arteries ligated prior to transplantation. Graph shows the mean values (± standard deviation).

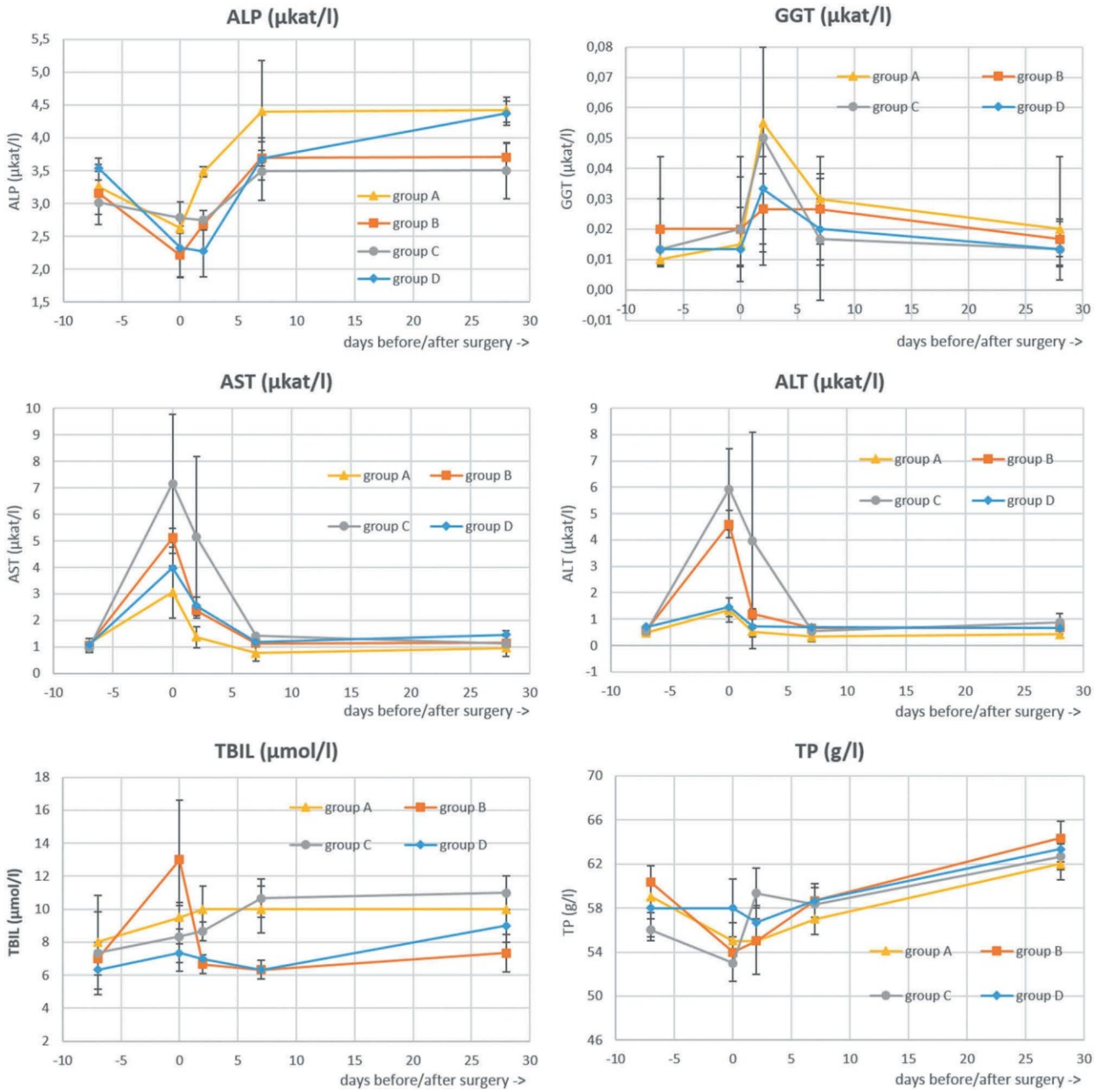

Figure 5. Changes in standard biochemical parameters of rats during the experiment. Serum was collected from rat tail vein 7 d prior the surgery, 4 h after the surgery, and then 2, 7, and 28 d after the surgery. Group A, B, and C – transplantation of 100, 500, and 1000 isolated pancreatic islets, respectively, with ligation of hepatic arteries; Group D – only ligation of hepatic arteries without islet transplantation. For all groups n = 6; graph shows the mean values (± standard deviation). ALP – alkaline phosphatase, GGT – gamma-glutamyltransferase, AST – aspartate transaminase, ALT – alanine transaminase, TBIL – total bilirubin, TP – total protein.

quantification of blood circulation defect can accurately reflect the extent and magnitude of portal vein micro-thrombosis and so the level of IBMIR early after islet transplantation.

In our study, we have combined the injection of MRI contrast agent into the tail vein with the ligation of hepatic arteries in order to prevent double perfusion of liver tissue from portal vein and hepatic arteries. First, we have tested the visualization of liver perfusion impairment using intravital dye patent blue or using MRI in combination with contrast agent. Due to double perfusion of liver through portal vein and hepatic arteries, the contrast agent is able to penetrate the ischemic liver tissue in a short time.

Some delay of hypo-perfused regions was visible (up to 2 min), but not detectable using MRI due to standard time needed for technical preparation of animal inside the scanner. Therefore, we proceeded to the ligation of hepatic arteries, making the portal vein the only blood supply for liver. Our results demonstrate that de-arterialized liver can survive with good function and without any side effects for at least 30 d. The biochemical values returned to the physiological range within 1 week after surgery. Animals healed the operating wound normally and increased the body weight in time in the same manner as healthy animals. This is consistent with published data reporting a good long-term function even in case of transplanted liver without arterial anastomosis in rat.[16,17] Using the interruption of the hepatic arterial blood flow, we prevented the intravenously injected contrast agent from penetrating the tissue behind the islets previously settled in peripheral branches of portal vein, thus enabling to "preserve" the areas of ischemic liver tissue for the subsequent imaging.

The ligation of hepatic arteries was followed by intraportal transplantation of pancreatic islets immediately and injection of patent blue dye 2 h later. Transplanted islets settle randomly in terminal branches of different liver lobes and patent blue clearly indicates the non-perfused regions, however, the quantification of ischemic tissue *in vivo* is impossible as it varies significantly inside the liver mass.

The MRI is considered as a safe, non-invasive method, which minimally burdens the patient and can be used repeatedly. Using proper coils and sequences in combination with gadolinium-based contrast agents, MRI can discriminate small lesions and enable even visualization of individual islets transplanted into liver of mice and rats and their localization in 3D reconstruction of whole animal.[19-26] The gadolinium-based contrast increases the signal intensity of tissue and enhances the sensitivity of the scanner to distinguish among enhanced (perfused), non-enhanced (= non-perfused), and tissues influenced with negative (iron-based) contrast agents.[27] Therefore, we concluded that this method should be sensitive enough for visualization of focal disturbances in liver perfusion.

In order to have fully developed thrombi at the time of measurement, the MRI procedure was started 2 h after islet transplantation (culmination of IBMIR). Using MRI sequences described above, the abdominal cavity was scanned and individual abdominal organs were visualized. Immediately after intravenous injection of contrast agent, regions with an enhanced signal are clearly distinguishable from those, which signal intensity remained on basic, non-enhanced level. It is possible to outline non-perfused liver regions and after multiplying by layer thickness to quantify the total liver volume as well as the volume of non-perfused tissue. Two days later, the ischemic necrosis is macroscopically visible in the same location where it was detected by MRI before. Using this experimental protocol, it is possible to detect the liver perfusion impairment immediately after transplantation of pancreatic islets into the portal vein *in vivo*.

The other set of experiments was focused on the sensitivity of this novel technique. The ultimate goal is the evaluation of therapeutic inhibition of IBMIR by various methods. To estimate the extent of ischemic liver tissue related to different levels of IBMIR intensity, we decided simply to transplant an increasing number of islets. Using the same experimental design, three groups of animal recipients were prepared and transplanted by 100, 500 and 1000 islets. The larger extent of non-perfused liver was expected and confirmed for a higher number of transplanted islets. The identical processing of data obtained from MR scanner was applied and the final extent of non-perfused tissue was recalculated to total liver volume. There was a significant difference in the extent of non- perfused liver tissue after transplantation of 100, 500 and 1000 pancreatic islets into the portal vein. In addition to that, the extent of non-perfused tissue correlates with a number of transplanted islets precisely (with small standard deviation between individual animals).

The study has several limitations, the first of all being its non-translatability into the clinical practice. Because of the ligation of hepatic arteries, it is impossible to use this model in humans. However, the main purpose of this study is an introduction of an experimental model, which allows comparing the efficiency of different experimental approaches focused on alleviation of IBMIR, which contribution to islet death after transplantation is indisputable and present in human as well as animal models. Thus, the transfer of this model into clinical practice

is not suitable nor requested as its main purpose is the evaluation of the therapeutic approach in general, but not the monitoring of each patient. The interruption of hepatic arteries prevents the method from the use even in large animals. Nevertheless, we believe that outcomes obtained using rodent models can considerably contribute to the improvement of islet pretreatment protocols and their establishment in routine clinical practice without the necessity of clinical trials based on this method. Although the microenvironment is very different in the case of rodent and human islet transplantation, the extent of liver ischemia serves in this case only as an "instrument" for demonstration/visualization of IBMIR intensity and its possible alleviation through the experimental interventions. Therefore, although this model cannot be directly transferred to clinical practice, it represents the only model for the evaluation of the effects of IBMIR-alleviating therapeutic interventions on liver perfusion *in vivo* and immediately after pancreatic islet transplantation.

Summarizing the presented results, we conclude that this model provides a unique and helpful technique for evaluation of the effect of different experimental interventions aimed at the inhibition of IBMIR *in vivo*. The main contribution of this study is the introduction of a visualization techni- que allowing the identification of liver ischemia extent in living animals without any detrimental effects on their health conditions.

4. Materials and methods

4.1. Study design

Isolated pancreatic islets in three different doses – 100 (Group A), 500 (Group B), and 1000 (Group C) – were transplanted into the portal vein of healthy rats (n = 6 in each group) with hepatic arteries ligated prior to transplantation. In Group D, only the ligation of hepatic arteries was performed. Two hours after islet transplantation (Group A, B and C), contrast-enhanced MRI of rat liver was performed and the volume of non-perfused liver tissue was quantified. Alternatively, for macroscopic evaluation, the distribution of intravital contrast dye patent blue (animals not included in experimental groups) was examined 2 h after islet transplantation. In order to confirm the safety of hepatic ligation, tail vein blood was collected for subsequent analysis 7 d prior the surgery, 4 h after the surgery, and then 2, 7 and 28 d after the surgery (all groups).

4.2. Animals

Male Brown Norway rats weighting 250–300 g (Velaz, Czech Republic) were used as both islet donors and recipients. All protocols related to this study were approved by the Animal Care Committee of the Institute for Clinical and Experimental Medicine and the Ministry of Health of Czech Republic. Experimental animals were maintained according to the European Convention on Animal Care in a controlled temperature, humidity, and 12/ 12 light/dark regimen with free access to food pellets and water. All surgical procedures were performed under total anesthesia induced by intramuscularly injected mixture of ketamine (Narketan, Vétoquinol S.A., France; 1,5 mg/100 g), dexmedetomidine (Dexdomitor, Orion Pharma, Czech Republic; 0,005 mg/100 g) and butorphanol (Torbugesic Vet, Zoetis, Spain; 0,05 mg/100 g).

4.3. Pancreatic islet isolation

Pancreatic islets were isolated using collagenase digestion followed by Ficoll discontinuous density gradient separation as previously described.[28] Briefly, pancreata of deeply anesthetized rats were filled through the bile duct with 15 ml of collagenase solution (Sigma-Aldrich, cat. # C9407; 1 mg/ml in HBSS, Hank´s balanced salt solution, Sigma-Aldrich, cat. # H8264). Excised pancreata were incubated for 10–15 min at 37°C with gentle shaking. Digested tissue was sieved through a 500 μm stainless steel mesh and islets were separated from exocrine tissue using Ficoll 400 discontinuous density gradient (Sigma-Aldrich, cat. # F9378; 1.108 g/ml, 1.096 g/ml, 1.069 g/ml, 1.037 g/ml). Purified islets were cultured overnight in CMRL-1066 (Biotech, cat. # P04-84600) based medium supplemented with 10% fetal bovine serum, 5% HEPES (both Sigma-Aldrich, cat. # F9665 and H0887, respectively) and 1% Penicillin/Streptomycin/L-Glutamine

(Thermo Fisher Scientific, cat. # 10378-016), at 37°C in 5% CO_2 atmosphere, and manually counted using dissection microscope prior to transplantation.

4.4. Liver ischemia model preparation

In order to increase the difference in MRI signal intensity of perfused and non-perfused liver tissue, the parallel arterial blood perfusion of the recipient liver was interrupted. Hepatic arteries (*arteria hepatica propria* and *arteria hepatoesophagica*) of Brown Norway rats were ligated (Figures 6 and 7) using Mersilk 7–0 non-absorbable material (Ethicon, cat. # W817) just before islet transplantation.

4.5. Pancreatic islet transplantation

After overnight cultivation, pancreatic islets (100, 500 or 1000) were transplanted into the portal vein

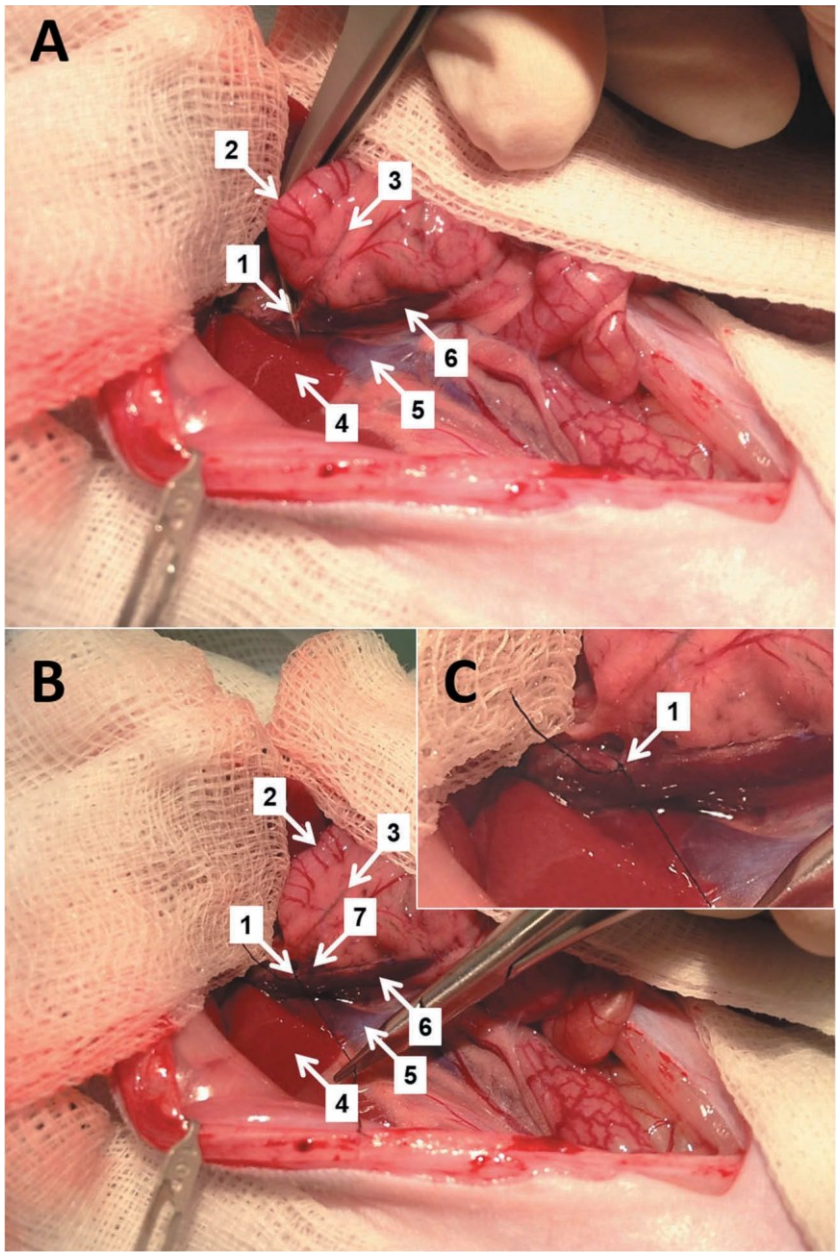

Figure 6. Preparation of *arteria hepatica propria* (A) and its ligation (B) in a detail (C). 1 – a. hepatica propria, 2 – duodenum, 3 – ductus choledochus, 4 – liver, 5 – v. cava caudalis, 6 – v. portae, 7 – v. pylorica.

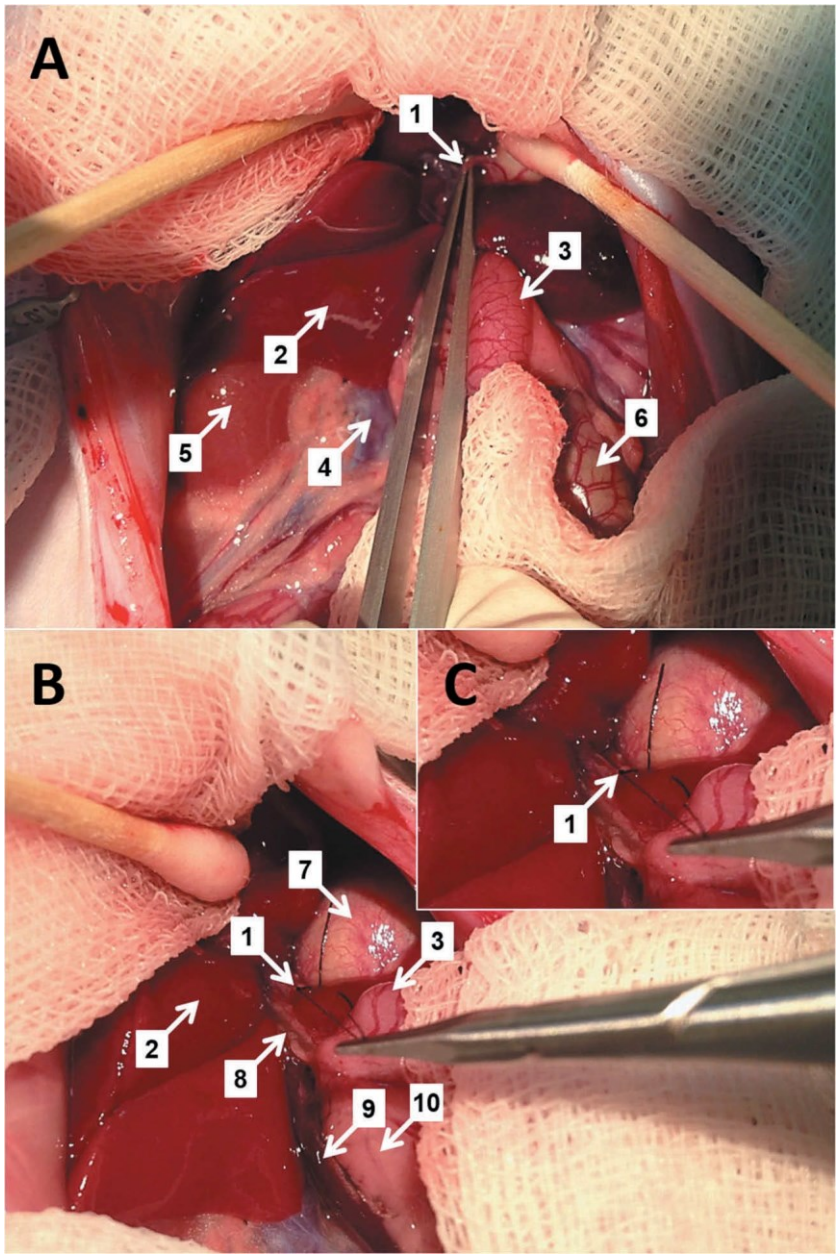

Figure 7. Preparation of *arteria hepatoesophagica* (A) and its ligation (B) in a detail (C). 1 – a. hepatoesophagica, 2 – liver, 3 – duodenum, 4 – v. cava caudalis, 5 – right kidney, 6 – cecum, 7 – stomach, 8 – ductus choledochus, 9 – v. portae, 10 - pancreas.

using standard technique.[29] Briefly, the midline laparotomy was performed in deeply anesthetized animal. Then, the large intestine was spread out off the abdominal cavity on a wet gauze in order to visualize the ileocecal vein. Islets were collected into the 27G butterfly catheter and injected into the ileocecal vein in total volume of 300 µl of saline. Bleeding was stopped by Avitene® flour (Bard Inc., cat. # 1010020) and mechanic press on the puncture. The abdominal cavity was then sutured in two layers using Vicryl 5–0 absorbable material (Ethicon, cat. # W9501).

4.6. Macroscopic visualization of intraportal thrombosis

One thousand of syngeneic pancreatic islets were transplanted into the portal vein of a healthy animal as described above, without ligation of hepatic arteries. Two hours after transplantation, the liver

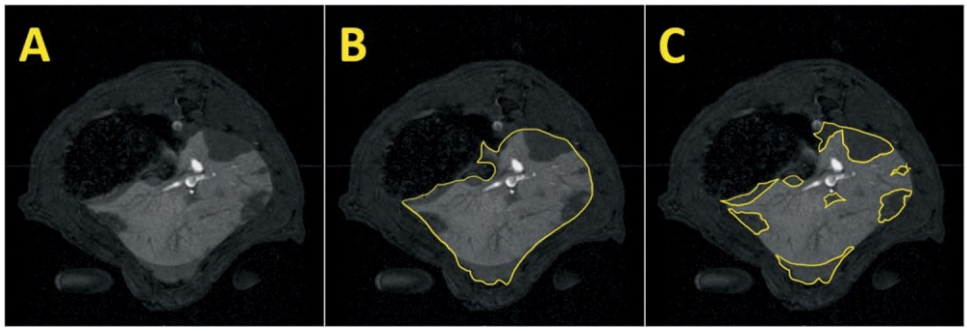

Figure 8. Illustrative picture of original magnetic resonance images of a rat abdominal cavity (A) with outlined liver (B) and ischemic areas within the liver tissue (C) after transplantation of 1000 syngeneic pancreatic islets using the MRI contrast agent to enhance the difference in the signal intensity of well-perfused and non-perfused liver tissue.

was excised, gently washed with saline and the portal vein tree was dissected and photographed. In another animal, the liver was examined macroscopically 2 and 28 d after transplantation.

4.7. Intravital staining with patent blue dye

For macroscopic evaluation of liver ischemia, 100 µl of intravital contrast patent blue dye (Bleu Patenté V, Sodique Guerbet 2,5%, France) was equally diluted in saline and injected into the portal vein 2 h after transplantation of 500 or 1000 pancreatic islet with or without ligation of hepatic arteries and a macroscopic picture was taken.

4.8. Magnetic resonance imaging

MRI was carried out on a 4.7 T Bruker BioSpec scanner (Bruker, Germany) using a resonator coil with a diameter of 7 cm (Bruker, Germany). For anatomical localization of liver tissue, T_2*-weighted MR images were acquired by a gradient echo sequence with the following parameters: repetition time (TR) = 111 ms, echo time (TE) = 3.7 ms, spatial resolution 0.254 × 0.254 × 1 mm^3 and scan time of 4 min. For imaging of ischemic lesions, MRI contrast agent MultiHance® (gadobenate dimeglumine, Bracco, cat.# 0270–5164; 0,1 mmol/kg) was administered into the rat tail vein through a catheter. Then, T_1 -weighted MR images of liver were acquired by a gradient echo sequence: TR = 94.5 ms, TE = 3.7 ms, spatial resolution 0.23 × 0.23 × 1 mm^3, number of acquisition (NA) = 16 and scan time of 6 min. Liver was usually covered by two stacks consisting of 11 slices.

4.9. Quantification of ischemic tissue volume

The volume of non-perfused liver tissue 2 h after transplantation was quantified based on magnetic resonance images using digital image analysis with ImageJ software (freely available at https://imagej.net/Welcome). Demonstration of the procedure is shown in Figure 8. Three trained experts evaluated pictures independently with the final result combining all three individual assessments.

4.10. Serum examination

To prove that ligation of hepatic arteries does not have any detrimental effect on animal health, rat blood was collected from the tail vein 7 d prior to, at day of (4 h after), and then 2, 7 and 28 d after the surgery in experimental groups A, B, C and D. The serum was prepared and examined for following biochemical parameters: alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), aspartate transaminase (AST) and alanine transaminase (ALT), total bilirubin (TBIL), and total protein (TP) level, using DRI-CHEM NX500i automated clinical chemistry analyzer (Fujifilm, Japan).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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