Glutamíne and ornithine alpha-ketoglutarate supplementation on malate dehydrogenases expression in hepatectomized rats[1]

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ABSTRACT

PURPOSE: To evaluate the relative gene expression (RGE) of cytosolic (MDH1) and mitochondrial (MDH2) malate dehydrogenases enzymes in partially hepatectomized rats after glutamine (GLN) or ornithine alpha-ketoglutarate (OKG) suplementation.

METHODS: One-hundred and eight male Wistar rats were randomly distributed into six groups (n=18): CCaL, GLNL and OKGL and fed calcium caseinate (*CCa)*, GLN and OKG, 0.5g/Kg by gavage, 30 minutes before laparotomy. CCaH, GLNH and OKGH groups were likewise fed 30 minutes before 70% partial hepatectomy. Blood and liver samples were collected three, seven and 14 days after laparotomy/hepatectomy for quantification of MDH1/MDH2 enzymes using the real-time polymerase chain reaction (PCR) methodology. Relative enzymes expression was calculated by the $2^{-\Delta\Delta C_T}$ method using the threshold cycle (C_T) value for normalization.

RESULTS: MDH1/MDH2 RGE was not different in hepatectomized rats treated with OKG compared to rats treated with CCa. However, MDH1/MDH2 RGE was greater on days 3 (321:1/26.48:1) and 7 (2.12:1/2.48:1) while MDH2 RGE was greater on day 14 (7.79:1) in hepatectomized rats treated with GLN compared to control animals.

CONCLUSION: Glutamine has beneficial effects in liver regeneration in rats by promoting an up-regulation of the MDH1 and MDH2 relative gene expression.

Key words: Glutamine. Ornithine. Hepatectomy. Liver Regeneration. Polymerase Chain Reaction. Enzymes. Rats.

Introduction

Potential mechanisms and pathways of liver regeneration have been extensively investigated in recent reviews[1,2]. Holecek[3] studied the effects of carbohydrates, lipids, and amino acids on partial hepatectomy and concluded that administration of a standard amino acid mixture without energy substrate has an inhibitory effect on liver regeneration. Birkhahn et al.[4] studies demonstrated that the inhibitory effect of glucose on liver regeneration can be diminished by the simultaneous administration of aminoacids and other substances.

Experimental studies have shown that ornithine alpha-ketoglutarate (OKG) supplementation of enteral feeding significantly shortens wound healing time in severe burn patients[5]. It has been reported that OKG stimulates the production on insulin and growth hormone promoting intracellular aminoacid transport and protein synthesis[6]. Glutamine (GLN) is the most abundant amino acid in the circulation and the main source of nitrogen for various metabolic processes including purine and pyrimidine synthesis necessary for DNA replication and cellular proliferation[7,8]. Yoshida et al.[8] treated partially hepatectomized rats with a parenteral solution containing GLN, resulting in increased uptake of the aminoacid by the hepatocytes and enterocytes and an increase in DNA and protein synthesis.

Two essential malate dehydrogenases, MDH1 (cytosolic malate dehydrogenase) and MDH2 (mitochondrial malate dehydrogenase), play important roles in the Krebs cycle for energy production through aerobic respiration[9]. MDH1 transports NADH equivalents across the mitochondrial membrane, controlling tricarboxylic acid (TCA) cycle pool size. MDH1 mRNA is expressed primarily in cardiac and skeletal muscle and in the brain, at intermediate levels in the spleen, kidney, intestine, liver, and testes and at low levels in lung and bone marrow[10]. MDH2 is the final enzyme in the mitochondrial tricarboxylic acid (TCA) cycle.

It catalyzes the inter-conversion of L-malate and oxaloacetate using nicotinamide adenine dinucleotide (NAD) as a cofactor to generate reducing equivalents[11].

Considering the importance of MDH1 and MHD2 in the metabolic processes and the absence of specific studies of the role of these enzymes in hepatic regeneration, this study aims to evaluate the expression of MDH1 and MHD2 in an experimental partial hepatectomy model after administration of OKG and GLN.

Methods

Approval for experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEUA, former CEPA) (protocol 54/08, 30 Sept 2008) and is in compliance with the Federal Law No. 11794 of October 8, 2008, and the Decree nº 6,689, July 15, 2009 that regulated the law in 11,794, available at: http: www.planalto.gov.brccivil03Ato2007-20102008LeiL11794.htm. The study was designed to minimize the number of animals required for the experiments.

In this controlled experimental study, after one week of acclimatization, 36 adult male Wistar rats weighting 170-325g, provided by the Sobral Zoonosis Center Vivarium were used. Rats were kept in a climate controlled room under 12-hour light/dark cycles with free access to food and tap water throughout the studies. Before the experiments the animals were randomly distributed into six groups (n =18) and treated (Table 1) as follows: groups CCaL, GLNL and OKGL were fed calcium caseinate (CCa), GLN and OKG, 0.5g/Kg by gavage, 30 minutes before laparotomy; the 3 remaining groups (CCaH, GLNH and OKGH) were likewise fed CCa, GLN and OKG, 0.5g/Kg by gavage, 30 minutes before 70% partial hepatectomy. Next, each group was divided into three subgroups (n=6) according to sample collection time (3, 7 and 14 days after laparotomy/hepatectomy) (Table 1).

TABLE 1 - Experimental groups and subgroups classified according to drugs used and surgical procedures.

Groups	Subgroups (n)			Drugs and Procedures
CCaL	CCaL3D (6)	CCa L 7D (6)	CCaL14D (6)	CCa + laparotomy
GLNL	GLN L3D (6)	GLN L 7D (6)	GLN L14D (6)	GLN + laparotomy
OKGL	OKG L3D (6)	OKG L7D (6)	OKG L14D (6)	OKG + laparotomy
CCaH	CCaH3D (6)	CCaH7D (6)	CCaH14D (6)	CCa + hepatectomy (70%)
GLNH	GLN H3D (6)	GLN H7D (6)	GLN H14D (6)	GLN + hepatectomy (70%)
OKGH	OKG H3D (6)	OKG H7D (6)	OKG H14D (6)	OKG + hepatectomy (70%)

CCa L - rats submitted to surgical trauma (laparotomy) receiving CCa; GLNL - rats submitted to surgical trauma (laparotomy) receiving GLN; OKGL - rats submitted to surgical trauma (laparotomy) receiving OKG; CCaH - rats submitted to 70% hepatectomy receiving CCa; GLNL - rats submitted to 70% hepatectomy receiving GLN; OKGL - rats submitted to 70%hepatectomy receiving OKG; 3D/7D/14D - samples collected on the 3rd, 7th and 14th day post laparotomy or 70% hepatectomy.

Drugs

Drugs used in the study were purchased from Nutrimed Industrial Ltda, Fortaleza, Brazil (Nutri Protein Nutrimed and L-glutamine), Chiesi S.A, Courbevoie, France (Cétornan - OKG), Promega Corporation, Promega Biotecnologia do Brasil, Ltda, Sao Paulo, Brazil (M-MLV Reverse Transcriptase), Life Technologies, New York, USA (Invitrogen™), Sigma-Aldrich Corp.(Sao Paulo, Brazil) *SyBr* Green™.

Surgical procedure

Anesthesia was induced with an intramuscular injection of ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg). The anesthetic regimen was the same throughout the experiment. At the end of three, seven or 14 days as scheduled, the animals were anesthetized for sample collection (1.0 ml of arterial blood and 1.0 g of liver tissue). Another small fragment of liver (150 mg) was individualized to be processed with specific reagents for analysis and quantification of malate dehydrogenase enzymes using the real-time PCR (polymerase chain reaction) methodology.

Laboratory procedures

Real-time PCR was used for quantification of malate dehydrogenase enzymes. In brief, 150 mg of liver tissue was homogenized in 1 ml of Trizol (Invitrogen™), compound containing phenol and guanidine thiocyanate. Next, 200 µl of chloroform was added to 1 ml of the homogenate. After centrifugation and separation of the phases, the RNA retained in the aqueous phase was transferred to another tube containing 500 µl of isopropyl phenol alcohol. After spinning, the liquid was discarded and the pellet (dehydrated RNA) was rehydrated with 1 ml of 75% ethanol and 50 µl of UltraPure™ DEPC-treated Water to 0.01 and stored at -80º C [12]. The production of cDNA was performed in 02 subsequent reactions. Reaction: 1 Oligo dT (Invitrogen™) (0.5 µg µl) -1 µL; Total RNA -1 µL; dNTP Mix (Invitrogen™) (1 mm) -10 µl; sterile H2O - 1 µL. Reaction 2: First-Stand Buffer 5 x-4 µl; Dithiothreitol (DTT) 0.1 M-2 µl; M-MLV Reverse Transcriptase 1 µL. The resulting solutions were stored at -20°C. As soon as the CDNA was defrosted, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), MDH1 e MDH2 (3.6µL) forward and reverse primers were added as well as other components (Sigma-Aldrich SyBr Green™ 10 µL, cDNA 10 ng e sterile H_2O 1 µL) before taking the mixture to the thermal cycler for initiation of PCR reaction[13,14]. Each test was conducted in triplicate and the final result was obtained by calculating the average of these results. The final values were obtained considering the calculations of delta threshold cycle (C_T) value $^{(\Delta C_T)}$ and delta/delta C_T $^{(\Delta\Delta C_T)}$ values[15,16]. Relative enzymes expression was calculated by $2^{-\Delta\Delta C_T}$ method using the threshold cycle (C_T) value for normalization[15,16]. The extracted RNA quantification was carried out spectrophotometrically GeneQuant Pro™ - Amersham Biosciences Corp NJ, USA) measuring the absorbance index at 260 nm and 280 nm wavelengths. Values greater than 1.6 were considered valid[17].

Statistical analyses

To ensure the appropriateness of parametric testing, all data were examined for normality, using Kolmorogov-Smirnov test (with Dalal-Wilkins Lilliefor P Value). Data (expressed as mean ± standard deviation) were examined for significance using ANOVA followed by Tukey multiple comparisons test. GraphPad Prism 4.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com) was used for statistical analysis and graphics design. Values of $p<0.05$ were accepted as statistically significant.

Results

MDH1 and MDH2 relative gene expression (RGE) was calculated by $2^{-\Delta\Delta Ct}$ method using the Ct value for normalization[15]. No differences were found in MDH1 RGE in rats submitted to laparotomy only when comparing the animals treated with CCa, GLN or OKG. Also, no significant differences were found in MDH2 expression in rats submitted to 70% hepatectomy and treated with OKG. However, these differences were greater in animals treated with GLN after three, seven and 14 days post-hepatectomy compared with rats treated with CCa. Calculated values for days three, seven and 14 are presented in Tables 2 and 3, Figure 1.

TABLE 2 - Liver enzyme MDH1 calculated values (ΔC_T, $\Delta\Delta C_T$) on the 3rd, 7th and 14th day of the experiment.

	3 days			7 days			14 days		
	ΔCt		$\Delta\Delta Ct$	ΔCt		$\Delta\Delta Ct$	ΔCt		$\Delta\Delta Ct$
Tx	LAP	HEP	HEP	LAP	HEP	HEP	LAP	HEP	HEP
CCA	-7.363	-1.368	5.995	0.012	-2.210	-2.222	-5.700	-7.956	-2.256
OKG	-2.375	-1.690	0.685	0.802	-1.757	-2.558	-0.653	-0.905	-0.251
GLN	-2.733	-5.093	-2.361	-1.414	-4.722	-3.307	-2.137	-5.099	-2.963

Tx = Treatment; CCA = Calcium caseinate; OKG = ornithine alpha-ketoglutarate; GLN = Glutamine; LAP= Laparotomy; HEP= Hepatectomy. The ΔCts were obtained by normalization with GAPDH gene. The $\Delta\Delta Ct$s were obtained using data from laparotomy animals as calibrator

TABLE 3 - Liver enzyme MDH2 calculated values (ΔC_T, $\Delta\Delta C_T$) on the 3rd, 7th and 14th day of the experiment.

	3 days			7 days			14 days		
	ΔCt		$\Delta\Delta Ct$	ΔCt		$\Delta\Delta Ct$	ΔCt		$\Delta\Delta Ct$
Tx	LAP	HEP	HEP	LAP	HEP	HEP	LAP	HEP	HEP
CCA	-7.475	-6.707	0.768	-3.885	-5.845	-1.960	-6.763	-6.617	0.146
OKG	-5.045	-2.322	2.723	-3.100	-1.882	1.218	-1.658	-3.201	-1.543
GLN	-4.735	-8.693	-3.958	-4.219	-7.492	-3.272	-3.325	-6.141	-2.816

Tx = Treatment; CCA = Calcium caseinate; OKG = ornithine alpha-ketoglutarate; GLN = Glutamine; LAP= Laparotomy; HEP= Hepatectomy. The ΔCts were obtained by normalization with GAPDH gene. The $\Delta\Delta Ct$s were obtained using data from laparotomy animals as calibrator.
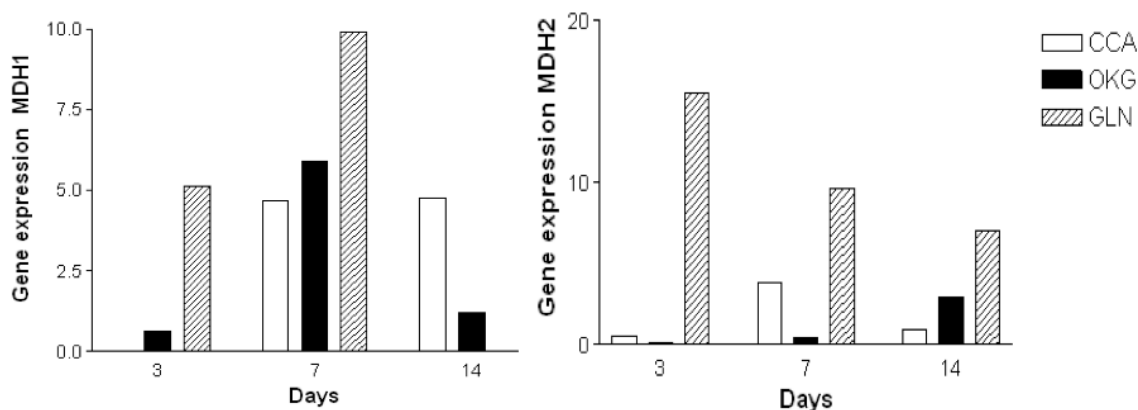

FIGURE 1 - Liver enzymes (MDH1, MDH2) relative gene expression ($2^{-\Delta\Delta C_T}$) on the 3rd, 7th and 14th day of the experiment. Results were obtained using the 2-ΔΔCT calculation (Livak and Schimittgen, 2001). MDH1 Relative Gene Expression (RGE) was greater on days 3 and 7, compared with CCA. MDH2 RGE was greater in all timepoints, compared with CCA in GLN-treated rats.

Discussion

Analysis of data from real-time quantitative PCR experiments may be carried out using different methods. The two most commonly used methods are absolute quantification and relative quantification. The first method (absolute quantification) determines the input copy number, usually by relating the PCR signal to a standard curve. The second method (relative quantification) relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control[15]. A reliable method to analyze the relative changes in gene expression from real-time quantitative PCR experiments is known as the 2-ΔΔCt. This method may be used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments[15].

According to Livak and Schimittgen[15], the endpoint of real-time PCR analysis is the threshold cycle of C_t. Considering that C_t is determined from a log–linear plot of the PCR signal versus the cycle number, the value obtained is an exponential and not a linear term. Therefore, any statistical presentation using the raw CT values should be avoided. Based on the assumptions, the 2^{-Ct} calculation was used here for results analyses.

Liver regeneration mechanism has been extensively studied. Hepatic cells are normally quiescent. However, after partial hepatectomy approximately 95% of hepatic cells rapidly re-enter the cell cycle. In the rat liver, the rate of DNA synthesis in hepatocytes begins to increase after about 12 hours and peaks around 24 hours. Non-parenchymal tissues have a later start for DNA synthesis.

Subsequent levels of DNA synthesis in hepatocytes are lower, as complete restoration of liver mass requires an average of ~1.6 cycles of replication in all cells[18]. Most of the increase in liver mass has occurred by three days after partial hepatectomy and mass restoration is complete in 5–7 days[19].

In order to maintain the metabolic homeostasis during regeneration the liver undergoes an adaptive response to regulate the differentiated functions of the hepatocyte. This is accomplished by the interplay between different sets of transcription factors, induced by the regenerative response[20]. Liver regeneration requires large amounts of energy to take place. One of the mechanisms involved in supplying energy to the hepatocyte is the autophagy seen in acute liver injury. However, hyperactivation of autophagy induces cell death, and the necrosis rate is actually a predictor of liver failure[21].

The malate/aspartate shuttle is the major pathway by which cytosolic reducing equivalents from NADH can enter the mitochondria and be oxidized[22]. MDH1 and MDH2 enzymes play important roles in the Krebs cycle for energy production through aerobic respiration[9]. The malate-aspartate shuttle translocates electrons produced during glycolysis across the semipermeable inner membrane of the mitochondrion to the electron transport chain via NADH to generate ATP. The shuttle system is required since the mitochondrial inner membrane is impermeable to NADH. To circumvent this, malate carries the reducing equivalents across the membrane[23].

This study has demonstrated that the use of GLN in partially hepatectomized rats promotes an over expression of MDH1 and MDH2 enzymes during liver regeneration. The explanation for the these findings is the same already duly proven in previous study[23] increased GLN availability enhances the malate-aspartate shuttle requiring an up-regulation of gene expression of both MDH1 and MDH2 enzymes.

The results presented here show that GLN supplementation in this model of partial hepatectomy has beneficial effects in liver regeneration considering that there was an up-regulation of the MDH1 and MDH2 gene expression in rats treated with GLN. On the other hand, the use of OKG did not lead to an overexpression of MDH1/MDH2.

Conclusions

Glutamine has beneficial effects in experimental liver regeneration by promoting an up-regulation of MDH1 and MDH2 gene expression. The use of ornithine alpha-ketoglutarate does not change MDH1 and MDH2 gene expression in experimental liver regeneration in rats.

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