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^{13}C-methionine breath tests for mitochondrial liver function assessment

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Abstract. – **^{13}C-methionine breath test has been proposed as a non-invasive tool for the assessment of human hepatic mithocondrial function. Two methionine breath labeled with ^{13}C in differents point of his molecular structure have been used for breath test analisys. Aim of this study was to compare two differently ^{13}C-labeled methionines in the evaluation of mitochondrial oxidation in basal conditions and after an acute oxidative stress.**

15 healthy male subjects (mean age 30.5 ± 3.1) received [methyl-^{13}C]-methionine dissolved in water. Breath samples were taken at baseline and and 10, 20, 30, 45, 60, 75, 90, 105 and 120 minutes after the ingestion of the labeled substrate. Forthy-eight hours later, subjects underwent the same test 30 minutes after ethanol ingestion (0,3 g/kg of body weight). Seven-day later, subjects underwent breath test using (L-methionine-1-^{13}COOH) as substrate, in basal condition and after ethanol ingestion.

At basal condition, the cumulative percentage of $^{13}CO_2$ recovered in breath during the test period (%cum-dose) was higher using L-methionine-1-^{13}COOH than [methyl-^{13}C]-methionine (10.25 ± 1.0 vs 4.07 ± 0.8; $p < 0.01$). After ethanol ingestion, % cum dose was significantly decreased at 60 and 120 minutes with both methionines (120 min: 10.25 ± 1.0 vs 5.03% ± 1.8; < 0.01 and 4.07 ± 0.8 vs 2.16% ± 0.9; $p < 0.01$, respectively). However, %cum-dose during L-methionine-1-^{13}C-breath test was significantly lower than that observed during methyl-^{13}C-methionine breath test (120 minutes: 5.03% ± 1.8 vs 2.16% ± 0.9; $p < 0.01$)

In conclusion, breath test based on L-methionine-1-^{13}COOH seems to show a greater reliability when compared to [methyl-^{13}C]-methionine to assess mitochondrial function because a larger amount of labeled carbon that reaches the Krebs' cicle.

Key Words:

Methionine, Breath test, Ethanol, Liver.

Introduction

Breath testing is a safe and non-invasive tool for investigating different metabolic pathways by means of ^{13}C-labeled substrates[1-5]. As far as liver is concerned, the evaluation of hepatic mitochondrial function in several liver diseases is an emerging topic in hepatology, although tests available are invasive and complex[6,7]. ^{13}C-Breath test using ketoisocaproic acid was proposed instead of more invasive tecniques[8]. Lauterburg et al evaluated mitochondrial liver function in healthy subjects after reversible manipulation of mitochondrial function induced by xenobiotics (ethanol, acetylsalicilic acid)[9]. The amino acid L-methionine is mainly metabolized by the liver and then oxidated by liver mitochondria, since most other tissues lack one or more of the enzymes involved in this process. The ^{13}C-methionine breath test based on Methyl-^{13}C-methionine (M-met test) has been recently used to study mitochondrial function after an acute oxidative stress in healthy subjects[10]. Methionine labeled in the carboxylic group (L-methionine-1-^{13}COOH) has been used to explore mitochondrial function in liver steatosis and in ethanol induced liver steatosis and in quantitative evaluation of liver function in early stages of liver transplantation in humans[11]. Therefore, two methionines labeled in different carbon-group have been proposed, but differences in the % of exhaled $^{13}CO_2$, in relation to different mitochondrial metabolic pathways of the ^{13}C-labeled molecule have never been tested. Aim of this study was to compare two different ^{13}C-labeled methionines [M-met and methionine labeled in the carboxylic group – L-Methionine-1-^{13}COOH (L-met)] in the evaluation of human mitochondrial oxidation by breath test analysis in basal condition and after a reversible impairment of liver mitochondrial function.

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Subjects and Methods

The protocol has been approved by the our Institutional Ethical Committee and the subjects gave the informed consent to the study.

15 healthy male subjects (mean age 30.5 ± 3.1 years; BMI 24.1 ± 0.8 Kg), social drinkers, went through the following steps:

1. after overnight fasting subjects, resting for 30 minutes prior to and during the test, received 1.5 mg/kg body weight of water-dissolved [Methyl-^{13}C]-methionine (99% atom isoenrichment, Isotec, Miamisburg, Ohio, USA) *per os*;
2. 48-hour later subjects underwent the same test after ethanol ingestion (0.3 g/kg body weight);
3. the following week subjects repeated the protocol (a+b), after receiving 1.5 mg/kg body weight of water-dissolved L-methionine-1-^{13}COOH (99% atom isoenrichment, Isotec, Miamisburg, Ohio, USA). Breath samples were taken at baseline and 10, 20, 30, 45, 60, 75, 90, 105 and 120 minutes after ^{13}C-methionine ingestion. $^{13}CO_2$ enrichment in the exhaled breath was analyzed through gas isotope ratio mass spectrometer (BreathMat, FinniganMat, Bremen, Germany). Results were expressed as: cumulative percentage of the dose of ^{13}C administered recovered in breath over test period (%^{13}C-cum dose), percentage of the dose of ^{13}C administered recovered per hour (%^{13}C-dose/h) and peak percentage of the dose of ^{13}C administered (^{13}C-peak). Results were expressed as mean ± SEM if not specifically indicated. Statistical analysis was performed using the Student's *t* test or Mann-Whitney U test for non parametric data where appropriate.

Results

At basal condition the ^{13}C-peak after the ingestion of M-met, was significantly earlier and lower than ^{13}C-peak after ingestion of L-met (mean ± SD: 30 ± 15 vs 60 ± 20 minutes, $p < 0.01$; mean ± SEM 2.99% ± 0.8 vs 7.9% ± 1.6; $p < 0.01$; Figure 1,2). The % ^{13}C-cum dose after 60 min amounted to 2.25 ± 0.4 for M-met and 3.84 ± 0.6 for L-met ($p < 0.05$), and to 4.07 ± 0.8 for M-met and 10.25 ± 1.0 for L-met after 120 min ($p < 0.01$; Figures 1, 3).

After acute ethanol intake the ^{13}C-peak, after the ingestion of M-met, was significantly earlier and lower than % ^{13}C-peak after ingestion of L-met (mean ± SD: 40 ± 15 vs 120 ± 30 min; $p < 0.01$; mean ± SEM: 1.60% ± 0.5 vs 3.89% ± 1.1; $p < 0.05$; Figures 2, 4).

The % ^{13}C-cum dose after 60 minutes was similar for both M-met and L-met breath tests (1.2% ± 0.3 and 1.6% ± 0.6, respectively; *p*: ns). Conversely, after 120 minutes the % ^{13}C-cum dose resulted higher using L-met (5.03% ± 1.8) than M-met (2.16% ± 0.9; $p < 0.01$; Figures 3, 4).

Finally, acute ethanol doses of 0.3 g/Kg body weight led to delayed and a significantly decreased of the ^{13}C-peak and of % ^{13}C-cum dose at 60 and 120 for both methionines (Figure 3).

Discussion

The great diffusion of isotope ratio mass spectrometry in Gastroenterology Units in the last decade for diagnosis of *Helicobacter pylori* by ^{13}C-Urea breath test allowed several investigators to perform many tests, based on carbon stable isotope to explore pancreatic, hepatic, intestinal and gastric functions[2-5]. In particular the ^{13}C-methionine breath test was used in animals to evaluate liver regeneration after hepatectomy and in human subjects to extimate mitochondrial toxicity due to severe valproic acid overdose and to explore hepatic function in alcoholic cirrhosis, in early stages of liver transplantation and hepatic

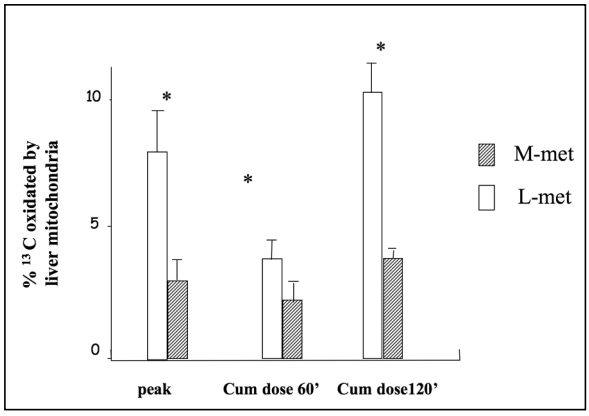

Figure 1. ^{13}C-peak, % cum dose 60 and 120 in basal conditions using both methionines. M-met: [methyl-^{13}C]-methionine; L-met: L-methionine-1-^{13}COOH. *$p < 0.01$.

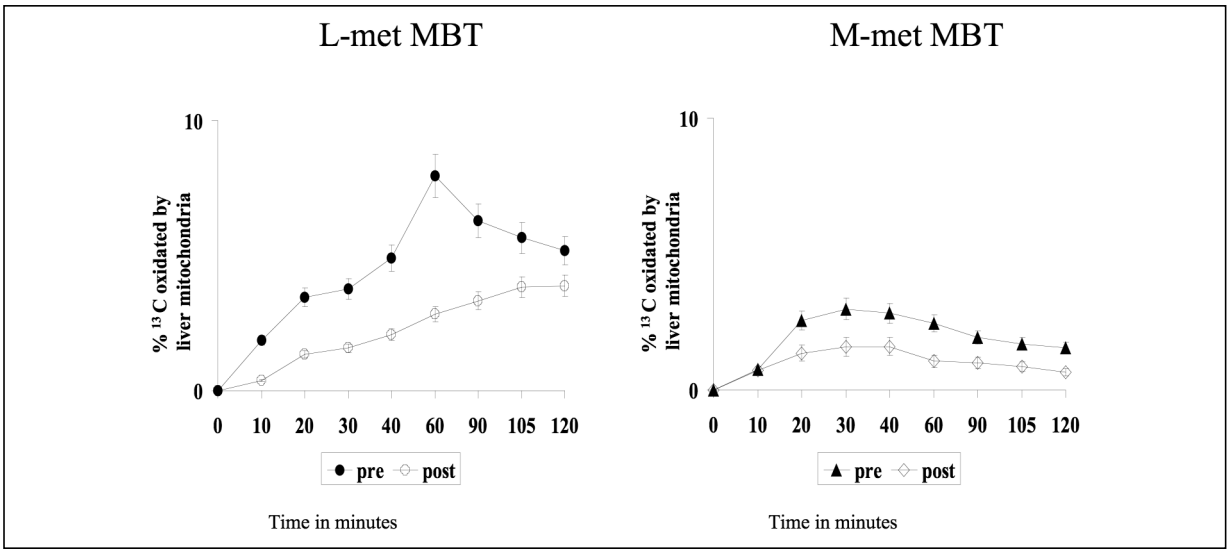

Figure 2. Percentage dose/hour of ^{13}C recovered in breath at different time point before and after ethanol oral load. L-met: L-methionine-1-^{13}COOH; M-met: [methyl-^{13}C]-methionine; L-met MBT: At any time $p < 0.01$; M-met MBT At 20, 30, 40, 60 minutes, $p < 0.05$.

mitochondrial oxidation in patients with pure non alcohol-related steatosis[11-15]. The present study explores the feasibility of two kinds of ^{13}C-methionine breath test in the evaluation of mitochondrial function.

Metabolism of methionine is complex. It depends on the availability of several aminoacids (serine, cysteine and methionine) and vitamins (vitamin B6, B12 and folic acid), other than on the genetic expression of several enzymes involved in his catabolism[16-17]. The main fates of methionine in humans are:

- production of polipeptidic chain;
- production of L-methionine-tRNA by methionine-tRNA synthetase;
- transmetylation to S-adenosyl-methionine (SAM) by methionine adenosyltransferase I.

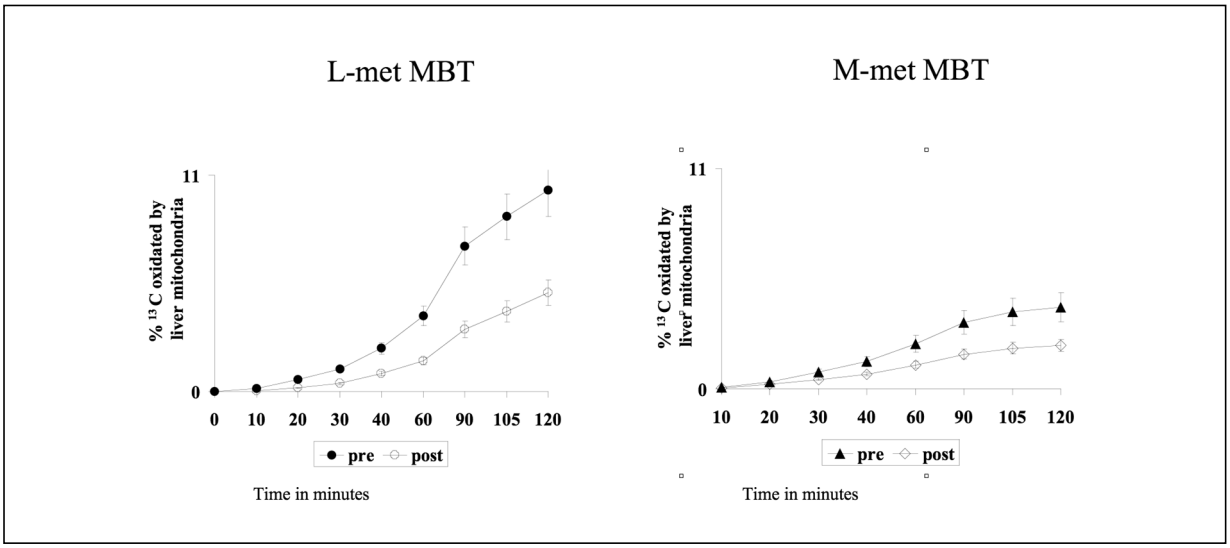

Figure 3. Cumulative percentage of ^{13}C recovered in the breath at different time point before and after ethanol oral load using both methionines. M-met: [methyl-^{13}C]-methionine; L-met: L-methionine-1-^{13}COOH L-met at 40 and 60 $p < 0.05$; at 90, 105, 120 minutes, $p < 0.01$; M-met at 90, 105 120 minutes, $p < 0.05$.

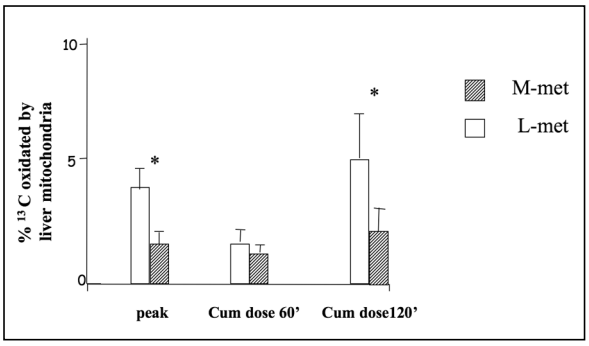

Figure 4. % ^{13}C-peak, % cum dose 60 and 120 after ethanol oral load using both methionines. M-met: [methyl-^{13}C]-methionine; L-met: L-methionine-1-^{13}COOH. * $p < 0.05$.

The first and the second pathways are sintetic and did not induce the production of CO_2. The latter, at the contrary, continues until production, via α-ketobutyrate, of odds free fatty acids or other glucogenic molecules and final production of exhalable $^{13}CO_2$[18]. Using the M-met, the ^{13}C was in the methyl group. The SAM is an universal donor of methyl groups, including DNA, RNA, hormones, neurotransmitters (epinephrine), membrane lipids (phosphatidyl choline), proteins, creatine, carnitine and many others. Although the labeled cabon atom is so widely spread in the organism and following his fate is impossible, a small part of labeled methionine methyl group is involved in production of sarcosine and it was finally detectable in breath as CO_2 molecules after further mitochondrial oxidation[18-20].

Conversely, the labeled carbon of L-met (carboxylic group) is part of the S-adenosylhomocisteyne after the methyl group of SAM was transferred to an acceptor. The ^{13}COOH after several steps may be incorporate in α-ketobutyrate. In mitochondria is present a specific α-ketobutyrate decarboxylase that produces $^{13}CO_2$[18-21].

The different fates of the labeled carbon explains the results we observed in the Methionine breath tests performed. A larger amount of labeled carbon of L-met compared to M-met seems to reaches the Krebs' cicle (into the mitochondrium) and then exaled in the breath. The impairment of mitochondrial function by ethanol load induces a reduction of recovery of $^{13}CO_2$ in breath in particular considering L-met. This result suggests that the metabolism of L-met is mitochondrial for a higher percentage than M-met.

In conclusion, ^{13}C-methionine breath tests could be proposed to non-invasively assess hepatic mitochondrial function and residual hepatic mitochondrial function after an oxidative stress. L-methionine-1-^{13}C breath test seems to measure the activity of a specific mitochondrial pathway (α-ketobutyrate decarboxylase) when compared to ^{13}C-methyl-methionine breath test. L-met breath test seems to better reflect hepatic mitochondrial function than M-met breath test both in basal conditions that after an acute ethanol induced oxidative stress.

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