

Original

# Amino acids change liver growth factors gene expression in malnourished rats

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## Abstract

**Background:** Glutamine and proline are metabolized in the liver and may collaborate on its regeneration. Parenteral nutrition (PN) containing either glutamine or proline was given to partially hepatectomized rats. The total RNA content and growth factor gene expression in hepatic remnants was measured, to determine the effects of these amino acid supplementation on the expression of growth factors during liver regeneration.

**Methods:** Wistar rats nourished (HN) and malnourished (HM) were hepatectomized and divided in two groups: 20 receiving PN enriched with Alanil-Glutamine (HN-Gln and HM-Gln) and 20 PN enriched with proline+alanine (HN-Pro and HM-Pro). The control groups comprised 7 nourished (CN) and 7 malnourished (CM) rats that didn't undergo surgery. Growth factor and thymidine kinase mRNA levels were measured by RT-PCR.

**Results:** In nourished rats, total hepatic RNA levels were lower in the HN-Gln and HN-Pro groups (0.75 and 0.63 µg/mg tissue, respectively) than in control group (1.67 µg/mg tissue) ( $P < 0.05$ ). In malnourished rats, total hepatic RNA content was higher in the HM-Pro group than HN-Pro, HM-Gln, and CM (3.18 vs. 0.63, 0.93 and 1.10 µg/mg, respectively;  $P < 0.05$ ). Hepatocyte growth factor mRNA was more abundant in the HM-Gln group when compared to CM (0.31 vs. 0.23 arbitrary units) and also in HM-Pro in relation to HM-Gln, HN-Pro, and CM (0.46 vs. 0.33 and 0.23, respectively,  $P < 0.05$ ).

**Conclusions:** Proline or glutamine supplementation in malnourished rats improves total RNA content in the remnant hepatic tissue. Amino acids administration increased HGF gene expression after partial hepatectomy in malnourished rats, with a greater effect of proline than glutamine.

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Key words: Amino acids. Parenteral nutrition. Liver regeneration. Proline. Glutamine.

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## LOS AMINOÁCIDOS ALTERAN LA EXPRESIÓN GENÉTICA DE FACTORES DE CRECIMIENTO HEPÁTICO EN RATAS MACHO DESNUTRIDAS

### Resumen

**Introducción:** La glutamina y la prolina de metabolizan en el hígado y pueden contribuir a la regeneración de este. Se administró nutrición parenteral con glutamina o prolina a ratas sometidas a hepatectomía parcial. Se midieron el contenido de ARN total así como la expresión genética del factor de crecimiento en el tejido hepático remanente, con el objetivo de determinar los efectos que provocaban estos aminoácidos en la expresión genética de factores de crecimiento durante el proceso de regeneración del hígado.

**Métodos:** ratas macho Wistar nutridas (HN) y desnutridas (HM) se sometieron a hepatectomía parcial y se dividieron en dos grupos: 20 recibieron nutrición parenteral enriquecida con Alanil-Glutamina (HN-Gln y HM-Gln) y 20 nutrición parenteral enriquecida con prolina+alanina (HN-Pro y HM-Pro). Los grupos de control estaban formados por 7 ratas nutridas (CN) y 7 desnutridas (CM) que no se sometieron a la cirugía. Los niveles de factor de crecimiento y timidina quinasa mRNA se midieron por RT-PCR.

**Resultados:** En las ratas nutridas, los niveles de ARN hepático total fueron inferiores en los grupos que recibieron HN-Gln y HN-Pro (0,75 y 0,63 mg/mg tejido, respectivamente) que en el grupo control (1,67 mg/mg tejido) ( $P < 0,05$ ). En las ratas desnutridas, el contenido total de ARN hepático fue superior en el grupo que recibió HM-Pro que el que recibió HN-Pro, HM-Gln y CM (3,18 vs 0,63, 0,93 y 1,10 mg/mg, respectivamente;  $P < 0,05$ ). El mRNA del factor de crecimiento de hepatocitos fue más abundante en el grupo que recibió HM-Gln con respecto a CM (0,31 vs 0,23 unidades arbitrarias), así como en HM-Pro con relación a HM-Gln, HN-Pro y CM (0,46 vs 0,33 y 0,23, respectivamente,  $P < 0,05$ ).

**Discusión:** La suplementación de prolina o glutamina en ratas macho desnutridas mejora el contenido total de ARN en el tejido hepático remanente. Al administrarse aminoácidos aumentó la expresión genética del HGF tras someter las ratas desnutridas a una hepatectomía parcial, obteniéndose un resultado mejor con prolina que con glutamina.

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Palabras clave: Aminoácidos. Nutrición parenteral. Hígado regeneración. Prolina. Glutamina.

## Introduction

Advances in surgical technique have increased the frequency of partial hepatectomy (PH) to remove malignant neoplasia.<sup>1</sup> Since the nutritional status of the patient is frequently compromised, a specialized nutrition during the perioperative period may improve the clinical course, support weight gain and reduce infectious morbidity.<sup>2,3</sup> Liver regeneration induced by hepatic resection occurs through hyperplasia and compensatory cellular hypertrophy of the remaining lobes.<sup>4</sup> It is controlled and mediated by intra and extra hepatic factors such as hormones, neurotransmitters, nutrients, proto-oncogenes, and growth factors.<sup>5,6</sup>

The objective of the present research is to identify the effect of glutamine and proline on the modulation of hepatic regeneration.<sup>6,7</sup> We have previously demonstrated in an identical experimental model that parenteral nutrition enriched with alanine-glutamine dipeptide increased the growth of remnant hepatic tissue and maintained normal morphology during hepatic regeneration in malnourished rats submitted to a 70% PH when compared with rats without hepatectomy.<sup>7</sup>

Proline is catabolized primarily in the liver, and when completely metabolized to glucose and urea, ATP is produced for gluconeogenesis and ureogenesis.<sup>8</sup> Until the present moment, it is not totally clear how glutamine could influence cellular metabolism and hepatic regeneration in nourished animals after partial hepatectomy.<sup>9-11</sup> In this study, we determine the effect of glutamine or proline enriched parenteral nutrition on the content of total RNA, hepatocyte growth factor (HGF), transforming growth factor- $\alpha$  (TGF- $\alpha$ ), thymidine kinase (TK) and mRNA levels in the hepatic remnants of well-nourished and malnourished rats.

## Methods

All experimental procedures were approved by the Ethical Research Committee (CAPPesq), School of Medicine, University of Sao Paulo, (Sao Paulo, Brazil). All animals received appropriate care throughout the experimental procedures.

### *Experimental protocol*

Male Wistar rats, mean body weight 250 g, from the University of São Paulo Medical School animal house (Centro de Bioterismo, FMUSP) were kept at room temperature with 12 hours light cycles. Ten days before the experiment, the rats were transferred to individual metabolic cages, with water ad libitum. Fifty four Wistar rats nourished (HN) and malnourished (HM) were hepatectomized and divided in two groups as previously described in an identical protocol.<sup>7</sup> Briefly, twenty rats that received parenteral nutrition enriched with Ala-Gln (10-HN-Gln and 10-HM-Gln)

and twenty rats enriched with proline plus alanine (10-HN-Pro and 10-HM-Pro). The control groups comprised seven nourished (CN) and seven malnourished (CM) rats that did not undergo surgery.

The nourished groups were fed with normocaloric (100 kcal/day) and normoprotein oral diet (14% of the total caloric value as casein) for ten days.<sup>12</sup> The malnourished group received a low-protein oral diet (4% of the total caloric value as casein) for ten days, until 10-15% of the initial body weight was lost.<sup>7</sup>

After these periods (nourished or malnourished), all animals were anesthetized with intraperitoneal injection of ketamine chlorhydrate (80 mg/kg of body weight) and submitted to jugular vein catheterization and partial hepatectomy (PH) under sterile conditions. Central vein cannulation was performed according to a standard technique. Rats were submitted to PH through 68-70% total liver tissue extirpation, including the median and left lateral hepatic lobes.<sup>13</sup> After PH, 3.0 mL of 10% glucose and 1.0 mL of 10% sodium bicarbonate solutions were intravenously injected in the animal.<sup>7</sup>

All experimental animals received isocaloric and isonitrogenic intravenous parenteral nutrition (PN) by a peritatic pump for 96 h. They were randomly selected to receive non-fat parenteral nutrition regimens containing 188 kcal/kg/day and 1.12 g N/kg/day, with 10% amino acid standard solution, supplemented with 10.4 g of L-alanyl-L-glutamine dipeptide (Alanine = 3.94 g and Glutamine = 6.46 g; Ala-Gln; Dipectiven®, Fresenius-Kabi-Germany) or a solution containing a mixture of 11.2g of alanine and proline (Alanine = 4.88 g and Proline = 6.32 g; Ala/Pro, Farmoterápica-Brazil). The experimental animals received an average of 5.14 g/kg/day of glutamine and 4.29 g/kg/day of proline.<sup>7</sup>

On postoperative day 4, the rats from HN and HM groups were anesthetized and laparotomized for extirpation of the total liver remnant. Liver samples were collected and frozen in liquid nitrogen.

The nourished and malnourished rats of the control groups (CN and CM) were not submitted to hepatectomy or catheterization. Four days after the adaptation period (10 days), with standard oral diet, control rats were anesthetized and laparotomized for total liver extirpation.

### *RNA extraction*

Total RNA was extracted from liver remnant as follows. Approximately 0.15 g of hepatic tissue liquid nitrogen powdered tissue was resuspended in 1 ml of lysis buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 100 mM  $\beta$ -mercaptoethanol), and homogenized. Total RNA was extracted with phenol-chloroform and isopropanol precipitation as described.<sup>14</sup> The total RNA concentration was obtained by spectrophotometry at 260 nm.<sup>14</sup> The concentration of total RNA, is reported as  $\mu\text{g}/\mu\text{L}$ , or  $\mu\text{g}/\text{mg}$  of hepatic tissue.

## RT-PCR analysis

Total RNA quality and integrity were assessed by electrophoresis on a formaldehyde-agarose gel.<sup>14</sup> All RNA samples were treated with DNase I (Amersham-Pharmacia) for 30 minutes at 37°C to eliminate genomic DNA contamination. One mg of DNase-treated RNA was used to measure thymidine kinase, HGF- $\alpha$ , TGF- $\alpha$  and mRNA. by RT-PCR with a Superscript One-step Platinum<sup>®</sup> Taq kit (Invitrogen<sup>®</sup>).<sup>15</sup>  $\beta_2$ -microglobulin (bMG) amplification served as an internal control for RNA quantization. Amplification in the absence of reverse transcriptase was a negative control. RT-PCR products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide<sup>16</sup>. The numbers of amplification cycles and the annealing temperatures used in the RT-PCR were as follows: HGF- $\alpha$  (26, 60°C); TGF (22, 55°C) and TK (28, 60°C).<sup>17,18</sup>

Primers based on rat sequences were designed for cDNA synthesis and for PCR amplification of the HGF, TGF- $\alpha$  and TK genes. The primer sequences and the predicted product sizes in base pairs (bp) were as follows: HGF sense 5'CCC GGT GCT GCA GCA TGT CCT3' and antisense 5'TCC CCT CGA GGA TTT CGA CAG3', 530 bp; TGF- $\alpha$  sense 5'GAC AAG TTG AAC AAG AAC CTC3' and antisense 5'CGT CAT CCA CCT AAT ACA TAA G3', 250 bp; TK sense 5'GCA GAT CCA GGT GAT TCT CG and antisense 5'ATC GAT GCC AAT GAC AGC CA3', 234 bp.<sup>16,18</sup>

## Statistical analysis

After densitometry measurement and normalization with  $\beta$ MG, each RT-PCR result was subjected to statistical analysis. Results were analyzed by nonparametric tests appropriate for the distribution and type of variables studied, using SPSS software. P values  $\leq 0.05$  were considered significant. Data are presented as the mean  $\pm$  SD, in arbitrary units.

Mann-Whitney test was used to compare the total hepatic RNA content and HGF, TGF- $\alpha$  and TK expression among the nourished and malnourished rats in each subgroup (Ala-Gln, Ala/Pro, and control without PH).<sup>19</sup>

Kruskal-Wallis method was used to compare levels of hepatic gene expression among the different subgroups (Gln, Pro, and control without PH).<sup>19</sup>

## Results

### Total RNA content

Partial hepatectomy (PH) and amino acid parenteral infusion influenced total RNA. The maximum percentage of total RNA among the experimental groups was: HM-Pro = 289% (3.18  $\mu$ g/mg hepatic tissue), HM-Gln

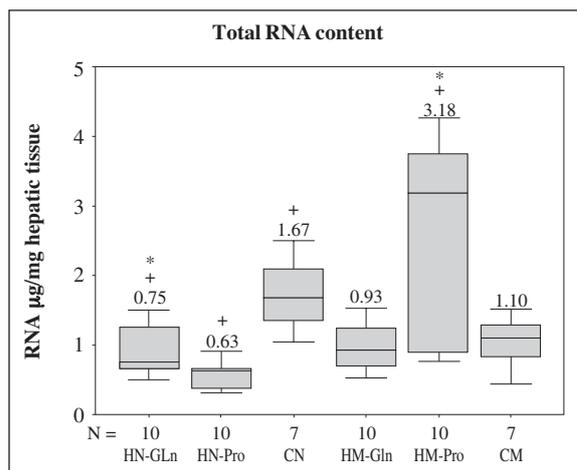


Fig. 1.—Median values of the total RNA content of the remaining liver. In relation to control group: HN-Gln and HN-Pro < CN; HM-Gln = CM; HM-Pro > CM and CN > CM (\*P < 0.05) and when comparing hepatectomized rats HN-Gln > HN-Pro; HN-Pro < HM-Pro; HN-Gln = HM-Gln; HM-Gln < HM-Gln (+P < 0.05).

= 84% (0.93  $\mu$ g/mg), HN-Gln = 45% (0.75  $\mu$ g/mg) and HN-Pro = 38% (0.63  $\mu$ g/mg). In relation to control group: HN-Gln and HN-Pro < CN; HM-Gln = CM; HM-Pro > CM and CN > CM (P < 0.05) and when comparing hepatectomized rats HN-Gln > HN-Pro; HN-Pro < HM-Pro; HN-Gln = HM-Gln; HM-Gln < HM-Gln (P < 0.05), as shown in figure 1.

### RT-PCR reactions

We performed semi-quantitative RT-PCR to determine the levels of HGF, TGF- $\alpha$  and TK gene expression.

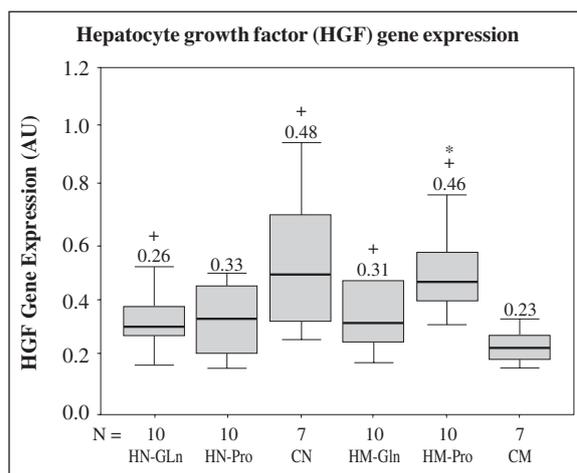


Fig. 2.—Median values of HGF gene expression of the remaining liver. Results normalized to  $\beta_2$ microglobulin and expressed in arbitrary units (AU). In relation to the control group: HN-Gln < CN; HN-Pro = CN; HM-Gln and HM-Pro > CM; CN > CM (+P < 0.05) and when comparing hepatectomized rats HN-Gln = HN-Pro; HN-Gln = HM-Gln; HM-Pro > HN-Pro; HM-Pro > HM-Gln (\*P < 0.05).

Different effects on HGF expression was observed after PH, when considering the animals' nutritional status. In malnourished rats, a significant increase in HGF expression was demonstrated, when compared to malnourished control group (HM-Gln = 0.31 and HM-Pro = 0.46 AU > CM, 0.23 AU, with  $P < 0.05$ ). In nourished animals, the opposite was observed and a significant reduction in HGF expression was demonstrated, when compared to nourished control group (HN-Gln = 0.26 and HN-Pro = 0.33 AU < CN, 0.48 AU, with  $P < 0.05$ ) – figure 2.

Malnourished rats, independently of the parenteral nutrition received, demonstrated higher HGF expression than nourished rats ( $P < 0.05$ ). Nevertheless, the nourished control group had significant more expression of HGF than malnourished control group (0.48 vs 0.23 AU, respectively, with  $P < 0.05$ ).

All the groups have TGF- $\alpha$  and TK positive expression, but without statistic difference between them.

## Discussion

The clinical results after PH depend on the regenerative liver process, liver resection extension, nutritional status, and per operative nutritional therapy.<sup>3</sup> The majority of patients considered candidates to hepatic resection may be malnourished and with protein depletion. Due to the depleted nutritional state, most of these patients are submitted to specialized nutritional therapy. As specific amino acids may have a particular role at the liver regeneration, it is worthwhile to study their effect in an experimental malnourished liver resection model, looking after specific changes in liver regeneration gene expression. We have previously observed that the hepatic regeneration index and hepatic growth percentage of malnourished rats subjected to PH using parenteral nutrition with glutamine or proline were higher than in control groups without hepatectomy.<sup>7</sup> The subsequent step was to study, in the same experimental model, the effects of parenteral glutamine and proline infusion on liver RNA content and hepatic regeneration gene.

In this study, our results have demonstrated that the nutritional status and the parenteral infusion supplemented with glutamine or proline could influence RNA content and gene expression involved in the hepatic regeneration. Parenteral nutrition supplemented with proline in malnourished rats (HM-Pro) protected against the reduction in content RNA, usually occurring after PH (fig. 1).

TGF- $\alpha$  and HGF are the main growth factors involved in the hepatic regeneration. HGF is considered to be the most powerful stimulator of hepatocyte proliferation, acting through paracrine and endocrine mechanisms to stimulate cellular mitosis. TGF- $\alpha$  probably participates in the later stages and acts through an autocrine mechanism.<sup>5</sup> TK levels are usually very low in normal hepatic tissue. However, 12 to 24 hours after PH, a significant increase is observed in this enzyme level

which remained elevated for 60 hours and declined to reach the baseline about 7 to 8 days. This event coincides with the greater cellular division activity, observed during hepatic regeneration.<sup>16</sup>

Therefore, we performed semi-quantitative RT-PCR to determine the levels of HGF, TGF- $\alpha$ , TK and mRNAs. The TGF- $\alpha$  and TK expression was verified for all groups with no statistical differences between them.

Our results demonstrate that 96 hours after partial hepatectomy, total RNA synthesis in remnant hepatic tissue from the nourished experimental groups (HN-Gln and HN-Pro) had still not been totally reestablished in relation to the liver RNA content in the intact controls, regardless of supplementation with glutamine or proline. However, a major tendency to approximate to basal values of total hepatic RNA was seen in groups HM-Pro and HM-Gln, suggesting that hepatic regeneration can be efficient, even under conditions of malnutrition.<sup>20</sup>

We found higher total RNA values in the malnourished group given parenteral nutrition (PN) supplemented with alanine plus proline, probably because the liver uses proline efficiently, even under conditions of amino acid deprivation.<sup>9,21</sup> Our results suggest that in the malnourished rats, supplementation of PN with proline probably favored the synthesis of total RNA during regeneration, possibly by triggering compensatory mechanisms for better use of the proline, with consequent economy in energy and amino acid use for transcription, protein synthesis, and proliferation.

The higher concentration of total RNA obtained in the HM-Pro group in relation to the HN-Pro group indicates a higher capacity for protein synthesis in the malnourished animals given PN enriched with proline. Proline might be related to the increase in total RNA, that reflects protein synthesis, and the possibility that this amino acid is being used in the production of collagen and not necessarily for hepatocyte proliferation.

Following PH, the genes involved in the expression of acute phase proteins and gluconeogenesis are quickly over expressed in order to restore metabolic homeostasis and to repair the tissue damage to the hepatic remnant.<sup>22</sup> An immediate cellular response to surgical trauma is fundamental to induce hepatocytes entering in the cell cycle.<sup>23</sup> Therefore our experimental model was not capable of demonstrating differences in TGF- $\alpha$  expression among the various experimental groups, probably because our samples were collected 96 h after partial hepatectomy, a point at which TGF- $\alpha$  mRNA levels have decreased gradually until they reached basal values, around the 6th postoperative day.<sup>17</sup>

We also found that HGF mRNA levels of HM-Pro group exceeded those of the HN-Pro group, while HGF expression in CM group was lower than in the CN group, suggesting that in these animals, the nutritional state and PH have a significant influence on HGF expression. In addition, proline administration increased

the HGF gene expression after partial hepatectomy in malnourished rats, more than glutamine. See figure 2 for more details.

In this study, we used the liver remnant, composed 90% of hepatocytes, for RNA extraction. Non-parenchymatous cells are the main secretors of HGF during hepatic regeneration<sup>24,25</sup> so, the use of total RNA origin from these cells as opposed to total RNA extracted from the residual liver, optimizes the amount of amplified HGF product, thereby improving the sensitivity of the method. This explains the significant differences between our results and those of Masson *et al* (1999), in which the RT-PCR reactions were carried out with RNA extracted from non-parenchymatous cells isolated from the residual liver.<sup>17</sup>

Since changes in TK gene expression can be related to growth and cell proliferation,<sup>16,26</sup> we measured TK mRNA levels 96 h after PH to determine whether TK over expression preceded a peak in DNA synthesis in this period, in order to identify the influence of parenteral infusion of amino acids on the later phases of hepatic regeneration. However, our results did not demonstrate any alterations in TK gene expression in this period in the various experimental groups, confirming that the increase in enzymatic activity up to 96 hours after partial hepatectomy does not occur in association with an increase in TK mRNA, which does occur earlier.<sup>17</sup>

Our results from nourished and malnourished hepatectomized rats submitted for 96 hours to PN supplemented with dipeptide Ala-Gln or Ala/Pro lead to the following conclusions: (i) hepatic total RNA levels were lower in nourished animals submitted to partial hepatectomy, independently of amino acid infusion; (ii) proline or glutamine supplementation in malnourished rats improves total RNA content in the remnant hepatic tissue; (iii) the administration of amino acids probably increased HGF gene expression after partial hepatectomy in malnourished rats, with proline having a greater effect than glutamine; and (iv) parenteral supplementation with glutamine or proline did not affect the levels of TGF- $\alpha$  and TK mRNAs in the hepatic remnant.

Finally, further studies are necessary to clarify the metabolic and molecular functions of amino acids, such as glutamine and proline, during hepatic regeneration under various nutrition conditions. These studies could involve the administration of nutrients, different models of hepatectomy, and the use of sensitive methods (in situ hybridization or microarray) to study the remnant hepatic tissue. The results of these studies could provide information on the safety and efficacy of the administration of amino acids during hepatic regeneration.

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