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LONG-TERM INTERMITTENT GLUTAMINE SUPPLEMENTATION REPAIRS INTESTINAL DAMAGE (STRUCTURE AND FUNCTIONAL MASS) WITH ADVANCED AGE: ASSESSMENT WITH PLASMA CITRULLINE IN A RODENT MODEL

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Abstract: *Objective:* Glutamine is the preferred fuel for the rat small intestine and promotes the growth of intestinal mucosa, especially in the event of gut injury. Quantitatively, glutamine is one important precursor for intestinal citrulline release. The aim of this study was to determine whether the effect of glutamine on the increase in intestinal villus height is correlated with an increase in both gut mass and citrulline plasma level in very old rats. *Methods:* We intermittently supplemented very old (27-mo) female rats with oral glutamine (20% of diet protein). Intestinal histomorphometric analysis of the small bowel was performed. Amino acids, in particular citrulline, were measured in the plasma, liver and jejunum. Markers of renal (creatinine, urea) and liver (alanine aminotransferase [ALT]) and aspartate aminotransferase (AST) functions were measured to evaluate renal and liver functions in relation to aging and to glutamine supplementation. Liver glutathione was also determined to evaluate cellular redox state. *Results:* Glutamine supplementation maintains the body weight of very old rats, not by limiting sarcopenia but rather by increasing the organ mass of the splanchnic area. Total intestine mass was significantly higher in glutamine-supplemented rats than in controls (15%). Measurement of villus height and crypt depth demonstrated that the difference between villus and crypt was significantly improved in glutamine pre-treated rats compared to controls (~ 11%). Plasma citrulline also increased by 15% in glutamine-supplemented rats compared to controls. *Conclusion:* Citrulline appears as a biomarker of enterocyte mass in villous atrophy associated with advanced age. Non-invasive measurement of this metabolite may be useful in following the state of the gastrointestinal tract in very old people, whose numbers are increasing worldwide and the care of whom is a major public health issue. The gut may contribute to the malnutrition caused by malabsorption frequently observed in the elderly.

Key words: Jejunum, histomorphometry, glutathione, creatinine, urea, rat.

Introduction

In senile rats, villous atrophy is found histologically, and scanning electron microscopy shows irregular architecture. These changes are essentially restricted to the proximal small intestine or the jejunum. Studies have shown that enzyme activity in the intestinal mucosa of senile rats is lower than in adults owing to the numerical reduction of the enterocytes (1, 2). A state of hyperproliferation has been observed in gastrointestinal epithelial cells (3) Gut mucosal kinetics changes with age and has lower rates of apoptosis and greater mucosal mass (4). For example, Schlafen 3, a novel negative regulator of growth, is markedly down-regulated in the colonic mucosa of aged rats (5). p53 can serve as a survival factor in tissues that respond to p53 activation by cell cycle arrest (e.g. endothelium of the small intestine) and so may play a role in the regulation of aging, in particular in longevity (6).

Glutamine is efficiently absorbed by the human jejunum in vivo (7). It has an important role in nitrogen homeostasis and intestinal substrate supply (8, 9). The intestines consume glutamine at a rate that is dependent on glutamine supply (10). Glutamine may also play a role in the gut-protective effect via down-regulation of the Sp3 gene (11) by inducing autophagy (12), by its anti-apoptotic effect on this tissue (13, 14), by modulating intestinal barrier function (15) under basal and stressed conditions in intestinal epithelial cells or by its anti-inflammatory effect via induction of the NF-kB p65 subunit (16). In humans, approximately 10-15% of glutamine taken up by the intestines is converted to citrulline (17, 18). Quantitatively, glutamine is considered as a major precursor for intestinal citrulline release (10, 19, 20) although its ability to be the source of citrulline carbon skeleton has been recently questioned (21). A good relation has been observed between the amount of metabolically active gut tissue and gut and body citrulline production (22-24).

In a previous study, we showed that glutamine supplementation of 5 months in very old female rats may fight against small intestine mucosa atrophy (25). The aim of the present study was to determine whether the effect of glutamine on the increase in intestinal villus height is correlated with an

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increase in gut mass and citrulline plasma level. Citrulline, as recently reported, is a reliable biomarker of functional intestinal mass in a number of pathological situations (17, 26-28).

We first evaluated the intestine mass in very old female Wistar rats supplemented or not with glutamine. We then measured villus height and crypt depth in enterocyte mucosa by histomorphometry and plasma levels of citrulline and other amino acids by amino acid analysis in both groups. We also tested oxidative status in very old rats supplemented or not with glutamine by measuring liver glutathione. In a preliminary experiment, we checked renal function in relation to age by examining the possible alterations in plasma creatinine and urea. In addition, to assess whether the effect of aging and the effect of glutamine supplementation both modify renal and liver functions in very old rats, we measured, in the main experiment, creatinine, urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Materials and methods

Materials

L-Glutamine used in supplementation experiments was purchased from Jerafrance (Jeufosse, France).

Animals

The experiments were performed in accordance with current legislation on animal experimentation in France. Wistar rats came from the experimental unit of the National Agronomic Research Centre of Theix, France. All animals were non-pregnant. They were housed in the animal facility until they reached the required age for the experiment. They were first acclimatized in cages in groups of five to a room with a 12h light/12h dark cycle (lights on at 8.00 AM) at 22°C. They were fed rat pellets (AO3 "growth diet" until the age of 10 months, and AO4 "maintenance diet" from 10 to 27 months). 27-mo-old rats corresponded to very old rats. The diets were purchased from Usine d'Alimentation Rationnelle (Villemoisson/Orge, France). The rats had free access to water and were fed ad libitum. All animals and experimental procedures were used in accordance with recommendations from the Institutional Ethics Committee of the Université de Clermont-Ferrand (France). Dominique Meynial-Denis and Luc Cynober are authorized to perform experiments on rats (# 04602 and 75-1472).

Experimental design

Preliminary experiment

The studies were conducted to assess the state of renal function, which may be defective with aging (29), in very old female Wistar rats. To determine the effect of aging on renal function, we examined the possible alterations in plasma creatinine and urea, which are specific markers of renal injury (30). Adult (10 month-old) and very old (27-month old) rats were used in this experiment (n=8 in each group). They were anesthetized with sodium pentobarbital (100μl / 100g body wt, intraperitoneally). Blood was taken from the abdominal aorta into a heparinized syringe to assay creatinine (PCr) and urea. The abdominal cavity was opened to remove the kidneys which were rinsed, wiped and weighed.

Main experiment

Old (27-month old) female rats, weighing ~ 350-400g, were used in this experiment. The animals underwent preliminary treatment for 10 months of their life span consisting in the addition of glutamine to drinking water for 7 consecutive days a month (20% of dietary protein - average of the 10 glutamine treatments, 0.8 ± 0.1 g/rat/d). Long-term intermittent treatment was used so as not to deteriorate renal function. Control rats received water only for the same period. All animals were weighed before and after glutamine supplementation. Very old rats were studied just after the last supplementation with glutamine, i.e. the day after glutamine supplementation ended. The animals were randomized into two groups: control old rats without supplementation (n=15) and glutamine-supplemented old rats (n=13). We suppressed three rats in the glutamine-supplemented group and one rat in the control group because their liver was abnormally big and the liver glutathione level was very low.

Isolation of rat jejunum, liver, spleen, kidneys and blood

Animals were anesthetized with pentobarbital sodium (100μl / 100g body wt) intraperitoneally. The abdominal cavity was opened and blood was collected by abdominal aortic puncture. The small intestine extending from the pylorus to the caecum was then removed. This tissue was emptied and rinsed with 150 mM NaCl solution. The gut was weighed. A 2-cm length taken in the proximal jejunum was cut and reserved for measurements of intestinal morphometry. A part of the remnant small bowel was used for amino acid analysis. The liver, spleen and kidneys were also dissected and weighed.

Intestinal histomorphometry

2 cm-intestinal jejunal samples were opened, promptly attached on a small cork plate, fixed in 10% formalin and then dehydrated and embedded in paraffin. 4μ sections were made and stained with hematoxylin-eosin-saffron. Histomorphometric analysis was performed to evaluate villous height and crypt depth in the jejunum with image analysis morphometric SAMBA(TM) IPS32 Version 4.7 software. This analysis was performed by a blinded investigator, as previously reported in (25, 31).

Perchloric extracts of plasma, jejunum and liver for amino acid analysis

Plasma was deproteinized with a 30% (w/v) sulfosalicylic acid solution and the supernatants were stored at -80°C until analysis. Tissues were ground and deproteinized with 10% trichloroacetic acid (TCA) containing 0.5 mM EDTA, and the

supernatants stored at -80°C until analysis. Amino acids were measured by ion exchange chromatography with ninhydrin detection after dilution of the samples with a lithium citrate buffer containing D-glucosaminic acid and amino-ethylcysteine as internal standards using an amino acid analyzer (AminoTac, JLC-500/V, Jeol, Tokyo, Japan) (32). The results of our participation in the European Quality Control Scheme (ERNDIM, Maastricht, the Netherlands) attest to the accuracy of our amino acid determinations.

Liver glutathione measurements

Liver was finely pulverized in liquid nitrogen and a sample was homogenized in 0.2M perchloric acid and 5 mM ethylenediaminetetra-acetic acid to measure total glutathione (reduced glutathione [GSH] plus oxidized glutathione [GSSG]) concentration. After centrifugation (8000g, 20min, 4°C), total glutathione content was measured in the supernatant as described by Mosoni et al. (33).

Plasma biochemical measurements

To assess the effect of aging on renal function, we examined the possible alterations in plasma creatinine and urea in a preliminary experiment. In contrast, in the main experiment, to determine whether glutamine supplementation modifies renal function and liver cytolysis in very old rats, we measured markers of renal (creatinine, urea) (30, 34), liver (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) alterations (30, 35). Plasma creatinine, urea, AST and ALT concentrations were determined by spectrophotometric assays on the Vista analyzer (Siemens®) following the manufacturer's recommendations.

Statistical analysis

Values are given as means ± SD. One-way ANOVA was performed to assess the effect of glutamine supplementation (treatment). When there was a significant effect, Student's t test (unpaired test) was used to compare two means. The level of significance was set at $P<0.05$.

Results

In a preliminary experiment, we verified, as previously observed (36), that the body weight of animals increased significantly with age (Table 1). Creatinine and urea in plasma were not different between adult and very old rats.

In the main experiment, we verified that all animals had the same initial body weight (BW); no significant effect of glutamine treatment on rat growth was detectable with advanced age (Table 2). There was no effect either on any tissue mass except that of the intestine, which was significantly higher in glutamine-supplemented rats (Table 2).

Table 1
Characteristics of adult and aged female Wistar rats: results obtained in a preliminary experiment

Variables	Adult rat (n=8)	Aged rat (n=8)
BW (g)	346±61	440±63*
Kidney mass (g)	1.2±0.1	1.3±0.3
PCr (μmol/L)	34.1± 3.8	35.4±8.1
Urea (mmol/L)	5.5±1.0	7.3±0.4

Mean values ± SD; *Significant ($p<0.05$); BW, body weight; PCr, plasma creatinine

Table 2
Effect of 10 month- glutamine supplementation on the body weight and on the mass of different tissues in very old female Wistar rats

Variables	Without glutamine (n=15)	With glutamine (n=13)
Initial BW (g)	349±47	356±44
Final BW (g)	385±65	373±48
Intestine mass (g)	8.0±1.5	9.1±1.7*
Liver mass (g)	9.6±1.6	10.6±1.8
Spleen mass (g)	1.1±0.4	1.2±0.3
Kidney mass (g)	1.1±0.3	1.3±0.3

Means ±SD; * Significant ($p<0.05$); BW; body weight

Effect of glutamine supplementation on plasma creatinine, urea, alanine transaminase and aspartate transaminase in very old rats

Plasma creatinine and urea were similar in very old rats supplemented or not with glutamine (Table 3). Likewise ALT and AST were not modified by glutamine supplementation (Table 3).

Table 3
Effect of 10 month- glutamine supplementation on the markers of both renal (PCr, urea) and liver (ALT, AST) functions in very old female Wistar rats

Variables	Without glutamine (n=15)	With glutamine (n=13)
PCr (μmol/l)	41±5	44±7
Urea (mmol/l)	6.6±1.2	7.7±3.2
ALT (UI/l)	41±15	40±17
AST (UI/l)	75±19	77±39

Means ±SD

Effect of long duration supplementation with glutamine on intestine histomorphometry

We decided to use the difference between villous height and crypt depth and not the villous height/crypt depth ratio, as in a previous study (25), should the wall of the villous and that of the crypt thicken at the same time with glutamine supplementation. Villus height and the difference between villus height and crypt depth were significantly higher in glutamine-supplemented rats than in controls (~10%), demonstrating that glutamine supplementation had a positive effect on the intestine (Table 4). In contrast, as mentioned above, the ratio villous height/crypt depth showed no significant difference between rat groups (Table 4).

Table 4

Effect of 10 month- glutamine supplementation on intestinal histomorphometry in female very old Wistar rats

Intestinal histomorphometry	Without glutamine (n=15)	With glutamine (n=13)
Villus height (μm)	651±80	719±90*
Crypt depth (μm)	198±27	216±35
Villus height / Crypt depth	3.23±0.29	3.36±0.27
Villus height – Crypt depth (μm)	452±62	503±61*

Means ± SD.*Significant ($p< 0.03$)

Effect of long duration supplementation with glutamine on amino acid levels in plasma, liver and intestine

Only the amino acids that were modified by glutamine supplementation are indicated in Tables 5, 6 and 7. In plasma (Table 5), both citrulline and proline levels were significantly higher in glutamine-supplemented rats than in controls. In contrast, glycine, serine and threonine were significantly decreased in the same group. However, glutamine levels were similar in both groups (without glutamine 691±101, with glutamine 703±100 μmoles/l). In the liver (Table 6), the level of citrulline like that of glutamine, serine, glycine, histidine and threonine was lower in glutamine-supplemented rats than in controls. In contrast, phenylalanine increased with supplementation. In the intestine, there was no change in the levels of citrulline nor in the other amino acids (Table 6).

Effect of long duration supplementation with glutamine on liver glutathione

In the liver, the glutathione level was not dependent on glutamine supplementation. The values obtained were the same in both groups of very old female rats: (6.2 ± 1.0 vs 6.1 ± 1.2 µmol/liver g, without and with glutamine, respectively).

Discussion

To our knowledge, the present study is the first to demonstrate that long-duration supplementation with glutamine in rats, as previously demonstrated with glutamine supplementation for 5 months of their life span (25), is efficient in increasing intestinal villus height in very old age. This increase was correlated with an increase in both gut mass and citrulline plasma levels.

Glutamine supplementation induced the production of citrulline and proline by the intestine (both citrulline and proline consist in end products of intestinal glutamine metabolism) and their release in the plasma (10, 37), in which hypercitrullinemia and hyperprolinemia were therefore observed. In contrast, only citrulline seemed to be consumed by the liver in glutamine-supplemented rats since its hepatic level decreased. Because citrullinemia was correlated with intestinal mass, in this paper we discuss only the relation between glutamine supplementation with advanced age and intestinal hypertrophy, as suggested by an elevated citrulline level. Citrullinemia is known to be greatly reduced when the enterocyte mass is reduced and in humans, the reduction is proportional to the severity of the intestinal disease. This observation may be extended to other situations in which intestinal function is compromised (17, 23). This is the case with advanced age, as reported by Thomson (38). In these conditions, citrulline may be considered as a conditionally essential amino acid, even if it is not a component of proteins (39). In our experiment, the regular supply of glutamine via glutamine supplementation over 10 months of life span to aged rats produced continuous release of citrulline from the small bowel into the plasma (increase of 15% in citrullinemia with glutamine supplementation). This increase in citrulline level was correlated with a similar increase in the villus height of the mucosa of glutamine-supplemented very old rats. It was also correlated with an increase in gut mass (11%). Consequently, because citrulline reflects an increase or a decrease in villus height in very old rats, it might be a biomarker of enterocyte mass in villous atrophy associated with advanced age.

Glutamine supplementation in very old rats did not modify creatinine and urea concentrations in the plasma. Consequently, since the rats in our experiment showed no evidence of significant renal failure, the circulating citrulline would be useful to monitor intestinal function, as observed by Crenn in humans (17, 23). In addition, no liver damage (no cytolysis) occurred with advanced age in our experiment because no changes in AST and ALT enzymes were observed. This is important to know if liver function is preserved because damaged liver releases arginase, thereby increasing the ornithine pool, which is the direct precursor of citrulline. In our experimental conditions, circulating citrulline appeared to originate only from the degradation of the glutamine supply. Because of the high turnover rate of glutamine, even large amounts (~ 6g one week a month/rat as previously reported (25)) can be given without any severe side effects (40). Another surprising point was that, unlike in a previous report (41), glutamine supplementation in very old rats did not modify the concentration of glutathione, of which glutamine is a known

Table 5
Effect of 10 month glutamine -supplementation on plasma amino acids in very old female Wistar rats.

Amino Acid (µmoles/l)	Without glutamine (n=15)	With glutamine (n=13)	P-value[a]
Threonine	227±40	194±47	0.059
Glycine	170±71	126±30	0.049
Serine	180±18	149±22	0.005
Proline	128±22	151±29	0.027
Citrulline	82±13	93±12	0.025

a. P-value for analysis of variance.

Table 6
Effect of 10 month- glutamine supplementation on tissue amino acids in very old female Wistar rats

	LIVER			JEJUNUM	
Amino Acid (µmoles/tissue g)	Without glutamine (n=15)	With glutamine (n=13)	P-value[a]	With glutamine (n=15)	Without glutamine (n=13)
Threonine	0.411±0.133	0.288±0.116	0.016	0.425±0.087	0.376±0.116
Phenylalanine	0.065±0.011	0.082±0.013	0.001	0.120±0.035	0.138±0.058
Glycine	1.331±0.424	0.907±0.258	<0.005	1.337±0.352	1.298±0.452
Serine	0.713±0.252	0.428±0.169	<0.005	0.399±0.083	0.362±0.127
Histidine	0.452±0.104	0.362±0.097	0.027	0.085±0.026	0.090±0.037
Glutamine	4.927±1.452	3.853±0.812	0.026	0.598±0.153	0.611±0.094
Citrulline	0.059±0.039	0.028±0.008	0.011	0.301±0.076	0.258±0.029

a. P-value for analysis of variance.

precursor. In other words, our findings provide evidence that glutamine supplementation preferentially enhances citrulline production by the intestine rather than glutathione production by the liver in very old rats. For this reason, it might be possible to use the known relation between citrulline and gut mass with advanced age in a rat model.

Conclusion

Our results suggest that long-term treatment with glutamine initiated before advanced age maintains rat body weight and has a beneficial effect on the enterocytes by increasing gut mass, improving the villus height of mucosa and thereby preventing the gut atrophy encountered in advanced age. Furthermore, the intact ability of enterocytes from very old rats - by preservation of mucosa enzyme activities for citrulline synthesis in the gut, as reported by Crenn et al. (23) - to continuously metabolize glutamine into citrulline allowed us, for the first time, to use citrulline as a noninvasive marker of intestinal atrophy induced by advanced age. Further investigations would be warranted to explore the effect of very old age on this glutamine-citrulline interrelation in the gut in vivo in humans. The widely documented occurrence of intestinal atrophy with advanced age (reduction of jejunal surface area) is a real public health issue because it may contribute to malnutrition in the elderly (anorexia of aging), which results in vulnerability to the frailty syndrome and increased mortality.

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