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Short Title:

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ABSTRACT.

Background: Glutamine is the major fuel for enterocytes and promotes growth of intestinal mucosa. Although oral glutamine exerts a positive effect on intestinal villus height in very old rats, how glutamine was used by enterocytes is unclear.

Methods: Adult (8-mo) and very old (27-mo) female rats were exposed to glutamine supplementation during 50 % of their age. Treated rats received glutamine added to the drink and control rats only water. Jejunal epithelial cells (~ $300x10^6$ cells) were incubated in oxygenated Krebs-Henseleit buffer for 30 min containing [1-^{13}C] glutamine (~ 17 M) for analysis of glutamine metabolites by ^{13}C NMR. An aliquot fraction was incubated in the presence of [U-^{14}C] glutamine for measuring produced CO_2. 98% of cells remained viable after 30 min of incubation. Biochemical reactions were stopped by addition of perchloric acid.

Results: Glutamine pretreatment increased glutamate production in very old rats (from NMR data). By contrast, this treatment decreased CO_2 production in very old animals. Possibly glutamate could be metabolized in the mucosa in first pass or in CO_2. The importance of each pathway was dependent of both age and glutamine pretreatment. The ratio CO_2/Glutamate, very high in control very old rats, became similar at both ages after glutamine pretreatment, as if very old enterocytes recovered metabolic capacities of adult enterocytes.

Conclusion: Our results suggest that, long-term treatment with glutamine started before advanced age: 1) it prevented the loss of rat body weight without limiting sarcopenia, 2) had a beneficial effect on enterocytes from very old rats by probably favoring the role of

glutamate as precursor for glutathione, arginine, and proline biosynthesis, not detected in ^{13}C NMR spectra in our experimental conditions. Further investigations would be warranted to explore these metabolic pathways.

Keywords:

^{13}C NMR, glutamine supplementation, histomorphometry, radiometry, jejunum

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Aging, an inevitable biological process, leads, in particular, to a progressive loss of muscle mass associated with a decline in strength or sarcopenia in humans and rodents (Young, 1990; Evans 1995; Dutta & Hadley, 1995; numerous inflammatory episodes (Yoshikawa, 1984; Papet, Dardevet, et al., 2003) that induce both a state of chronic, continuous stress (as reported by Mayer et al., 2001 in general process of stress) and a general decline in the physiological function leading to morbidity and mortality. There is generally a decrease of function of the gastrointestinal tract, but there may be loss of adaptability in response to changes in diet or nutritional stress (Drozdowski and Thomson, 2006). Pathological alterations which might lead to minor overall intestinal functional variations in the youth because of a process of adaptation, may lead to much more serious events in the elderly.

The reduced mass of muscle, in the elderly may limit the availability of glutamine (muscle being important glutamine producer in the whole body), which is essential for immune cell function (Munro, 1982; Young, 1990; Mondello et al., 2010) and intestine oxidative fuel (Curi et al., 2005) in animals and humans. Moreover, glutamine has enterocyte-protective effects and modulates intestinal barrier function in stressed animals and humans as well as laboratory models (Amasheh et al., 2009; Hering and Schulzke, 2009). Although recent findings on glutamine concerned essentially ill humans (Wischmeyer, 2003; Fuchs and Bode, 2006; …), few data were reported in very old female rats. For this reason, glutamine supplementation was used in such animals in order to study the benefit of glutamine on intestine metabolism in very old healthy animals.

In the current study, the hypothesis that glutamine is degraded by small intestine in glutamate and other carbon metabolites was tested by using ^{13}C nuclear magnetic resonance (NMR) to trace the metabolism of [1-^{13}C] glutamine into enriched isotopomers of glutamine products degradation. As glutamate and CO_2 were nevertheless major metabolites from glutamine, we used radiometry to follow CO_2 production during

incubation of proximal intestine. Before the experiment, adult (8-mo) and very old (27-mo) female Wistar rats were exposed to glutamine supplementation during 50% of their age, for 7 days in a row a month as previously described (Mignon et al. 2007a). These data demonstrated that glutamine pretreatment increased labeled glutamate production by isolated enterocytes, only in very old rats but decreased CO_2 production in both adult and very old rats.

MATERIALS AND METHODS

Materials

L-Glutamine used in supplementation experiments was purchased from Jerafrance (Jeufosse, France). [1-^{13}C] Glutamine was bought from Eurisotop, (Saint Aubin, France). [^{14}C]Glutamate (262 mCi/mmol) and [U-^{14}C] Glutamine (255 mCi/mmol) were purchased from Amersham (Bucks, UK).

Animals

These experiments were performed in accordance with current legislation on animal experimentation in France. Wistar rats originated from the experimental Unit of the Research Center of Theix. All animals were no pregnant. Rats were housed in the animal facilities until they reached the required age for the experiment. Animals were acclimatized in cages in groups of 5 in a room with a 12 h light/12 h dark cycle (lights on at 8.00 AM) at 22 °C. The rats were fed rat pellets (AO3 "growth diet" until they attained 10 months, and AO4 "maintenance diet" from 10 to 27 months). These diets were purchased from Usine d'Alimentation Rationnelle, Villemoisson/Orge, France. Rats had free access water and were fed ad libitum.

Experimental design

Adult (8-month aged) and old (27-month aged) female rats, weighing ~ 350-400 g, were used in this experiment. Animals were exposed to preliminary treatment during 50 % of their age i.e glutamine supplementation by the addition of glutamine to the drinking water for 7 days in a row a month (20% of dietary protein). It consisted in an intermittent treatment of long duration in order not to deteriorate the renal function which can already be defective in the very old animals (Walrand et al., 2008). Control rats received only water during the same period. Animals were weighed before and after glutamine supplementation in order to demonstrate if glutamine supplementation was efficient to improve animal body weight. Adult and very old rats were studied about fifteen days after the last supplementation with glutamine. Rats were randomized to the following groups. Four groups of animals were used in this experiment: 1) control adults without supplementation (n=10); 2) glutamine-supplemented adults (n=10); 3) control old rats without supplementation (n=10); 4) glutamine-supplemented old rats (n=10).

Isolation of jejunal epithelial cells and incubation

Animals were anesthetized with pentobarbital sodium (100µl / 100g body wt, intraperitoneally). The abdominal cavity was opened and the small intestine extending from the pylorus to the caecum was removed. This tissue was emptied and rinsed with 150 mM NaCl solution. A 2-cm length taken in proximal jejunum was cut and reserved for measurements of intestinal morphometry. The remnant small bowel was used for enterocytes isolation. Indeed, jejunal epithelial cells were prepared under continuous gas (oxygen/carbon dioxide, 19/1) and resuspended in bicarbonate Krebs-Henseleit buffer (pH7.4), as described previously (Vidal et al, 1988; Blachier et al, 1991; Lardy et al, 2004). The cell density of the final suspension was assessed by account of an aliquot in a

Malassez hematocytometer. Cell viability was evaluated by the Trypan blue exclusion test and by the percentage of lactate deshydrogenase that was released in the extracellular medium at the onset of incubation (time 0) and after 30 min of incubation. At the end of incubation, 98% of cells were viable. For measurement of glutamine metabolites by ^{13}C NMR, about $300x10^6$ cells were resuspended and incubated in 6ml of oxygenated Krebs-Henseleit for 30 min (37°C) containing ~ 17 mmol/l of [1-^{13}C] glutamine. A supraphysiological level of glutamine was chosen to induce glutamine degradation and to be easily detected by NMR (Cremin et al., 1997). An aliquot fraction (25x106 cells) was taken and incubated, in parallel, in the presence of 1 nmol of [U-^{14}C] glutamine (0.25µCurie) for the measurement of produced CO_2. The incubation was stopped by addition of 30% perchloric acid to obtain cell suspension in an acidified incubation medium at 10% of perchloric acid.

Perchloric extracts of enterocytes for NMR studies

The acidified cell suspension was kept in ice for 10 min to precipitate proteins and then centrifuged for 10 min at 12 000 g at 4°C. The supernatant was decanted and neutralized with KOH 5M. Then it was kept in ice for 10 min and centrifuged for 10 min at 12 000g at 4°C to rule out the KClO4 precipitate. The supernatant was removed and frozen at -20°C until freeze-drying. The obtained sample was redissolved in 800 µl of 99.9% D2O containing 3mM EDTA and 20mM cycloleucine. Proceed by successive weightings because cycloleucine was used as both internal standard and quantification standard.

^{13}C NMR spectroscopy

The13C NMR experiments were performed at 100.625 MHz using the DRX 400 Bruker spectrometer (Ettlingen, Germany) equipped with an heteronucleus probe. Spectra were acquired at 298K with hard pulse of 5.5 us length to perform 90°. Each spectrum

represented an average of 3500 scans with recycle time of 5.3 s. A sweep width of 23148k and 16 k memory size were used. A WALTZ-16 composite pulse-decoupling sequence were applied just during the acquisition time to avoid the Nuclear Overhauser Effect. To analyse NMR data, the FIDs were processed with a 1-Hz line broadening before Fourier transformation. Chemical shifts are given relative to an internal cycloleucine standard set at 26 ppm.with a pH adjusted at 7.4, as reported by Badar-Goffer et al., 1990.

Calculation of Glutamate production from [1-^{13}C]Glutamine

Glutamate produced was quantified by comparison with cycloleucine added in NMR tube to obtain a concentration of 20 mM.

$$[^{13}\text{C Glu}] = \frac{\text{Area Glu}_{(C2+C3+C4)}/3) \times [\text{RI}]}{(\text{Area RI}/4)} \qquad (1)$$

where area $\text{Glu}_{(C2+C3+C4}$ et area RI represented the sum of measured areas for the three carbons 2,3 and 4 of Glu ^{13}C and those of 4 carbons of cycloleucine used as internal reference; these areas were respectively divided by 3 and 4 to be reported to an individual carbon, [RI] was the concentration of cycloleucine in NMR tube in mM. [^{13}C Glu] was also expressed in mM.

•The total amount of ^{13}C Glu produced in an assay by the total quantity of incubated enterocytes during 30 min in the présence of ^{13}C Gln was:

$$\text{qGlu} = \frac{[^{13}\text{C Glu}] \times V}{1000} \qquad (2)$$

where V was the necessary volume to redissolve the freeze-dried sample in µl.
qGlu was expressed in µmol.

•Production of Glu in nmol by million of enterocytes by incubation minute

$$\text{Glu produit} = \frac{\text{qGlu}}{N} \times \frac{1000}{30} \qquad (3)$$

where N was the number of enterocytes millions in a given incubation, 30 was corresponding to 30 min of incubation and 1000 was the factor to convert µmol into nmol.

Intestinal histomorphometry

2 cm-intestinal jejunal samples were opened, promptly attached on a small cork plate and fixed in 10% formalin 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 jejunum with image analysis morphometric SAMBA(TM) IPS32 Version 4.7 software. In case the wall of the villous and that of the crypt would thicken at the same time with Gln supplementation, we reported the difference between villous height and crypt depth and not the ratio villous height on crypt depth, as previously published (Mignon, Beaufrère, JPEN 2007a)

Statistical analysis

Values are given as means ± SD. Variance analyses were performed to discriminate among the effects of aging (A), supplementation (S) and their interactions. Comparisons between 2 means were carried out using Student's *t* test (unpaired test). The level of significance was at $p<0.05$.

RESULTS

Characteristics of animals

Adult animals were young at the beginning of glutamine supplementation. For this reason, they grow during the treatment with or without glutamine supplementation (~ 15%). By contrast, glutamine supplementation has a beneficial effect on the body weight of very old rats; this treatment prevents the loss of weight due to the age (equivalent to ~ 15% in animals without glutamine treatment) (fig 1). By contrast, glutamine supplementation did not stop sarcopenia (by example, tibialis mass was not improved by this supplementation: ~1.245g in adults vs 1.003g in aged rats, regardless of treatment).

Effect of long duration supplementation with glutamine on glutamate production from isolated and incubated enterocytes

A representative ^{13}C NMR spectrum of a glutamine-supplemented incubation in adult enterocytes was presented in fig 2. This spectrum was representative from both adult and very old enterocytes because detected peaks were similar whatever the age of animals. By contrast, peak integration of Glu (carbon C2, carbon C3, carbon C4) allowed to evidence differences in produced labeled Glu quantity. From these data, it was possible to calculate Glu production from [1-^{13}C] Glutamine; these results were reported in the figure 3. It should be pointed that only part (A) of the spectrum was used to quantify Glu because integration of COOH resonances may be disturbed by the huge peak of the remaining precursor, [1-^{13}C] Glutamine. Long duration supplementation with glutamine before enterocytes preparation from adult and aged rats had no effect in adult enterocytes but significantly increased Glu production in very old enterocytes: a rise of ~ 50% was observed. Indeed, the obtained value in NMR spectra increased from 0.43±0.13 to 0.64±0.21 nmol/10^6 viable cells/min.

Effect of long duration supplementation with glutamine on CO_2 production from isolated and incubated enterocytes

CO_2 production from adult and old incubated enterocytes in the presence of [U-^{14}C] Glutamine was presented in fig 3. CO_2 production was higher in old than adult rats. However, CO_2 production was always significantly smaller in glutamine-supplemented rats whatever their age.

Effect of long duration supplementation with glutamine on intestine histomorphometry (Table 1).

By measuring the villus height and the crypt depth and calculating the difference, we failed les linéaments to demonstrate an effect of Gln supplementation on intestine, contrary to previous results obtained after Gln supplementation (Mignon, Beaufrère, JPEN 2007a).

DISCUSSION

To our knowledge, the current study is first to demonstrate that long duration supplementation with glutamine allowed us to differentiate glutamate and CO_2 production from glutamine in isolated enterocytes in relation with age. This supplementation had, as previously reported Mignon et al, 2007, prevented the loss of body weight in very old rats without stopping sarcopenia.

A surprising point was that there was no change in both the value of villus height and this of crypt depth, nor even in the difference between villus height and crypt depth after long term treatment with glutamine. This was not in good agreement with results that we previously reported (Mignon, Beaufrère, 2007a). However, the difference between these two experiments was the time choice of Gln supplementation stop. In this experience, adult and very old rats were studied about fifteen days after the last supplementation with glutamine whereas previously, they were sacrificed just at the end of treatment. Because of Gln may be intensively used by gut to produce citrulline, as reported by Rutten et al. (2006) after Gln supplementation in catabolic state so that the supply of glutamine could be insufficient after 15 days of treatment disruption. Consequently, continuous supply of Gln may be necessary to compensate as better as possible these impaired metabolic pathways.

Moreover, the Gln supply effect was not long-lasting so much Gln requirements must be intense with advanced age.

The findings of this study evidenced by ^{13}C NMR, the efficiency of long duration supplementation with glutamine to increase labeled glutamate production in very old rats. By contrast, the ratio CO_2/Glutamate became similar in adult and aged enterocytes at the end of incubation (0.606 ± 0.165 in adults vs 0.507 ± 0.279 in aged rats) although this ratio was significantly different without glutamine supplementation (0.803 ± 0.381 in adults vs 1.021 ± 0.386 in aged rats, $P<0.01$). Everything takes place as if CO_2 production that is the main metabolic fate of glutamate within enterocytes and furnish energy to enterocytes (Blachier et al., 2009) became minor after glutamine supplementation whatever the age of animals. Because of glutamine supplementation maybe not improve protein synthesis rate in the jejunal mucosa (Ferreira S. Tannus et al., 2009), glutamate can be used by enterocytes to produce other amino acids L-aspartate, L-alanine, L-proline, L-ornithine and L-citrulline (as reported by Fleming et al., 1997) and glutathione, a crucial antioxidant in gastrointestinal. But these metabolites are not detectable by NMR in our experimental conditions.

In summary, our results suggest that, long-term treatment with glutamine started before advanced age: 1) it prevented the loss of rat body weight without limiting sarcopenia, 2) had a beneficial effect on enterocytes from very old rats by favoring the role of glutamate as precursor for glutathione, arginine, and proline biosynthesis, not detected in ^{13}C NMR spectra in our experimental conditions. Further investigations would be warranted to explore these metabolic pathways.

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FIGURE LEGENDS

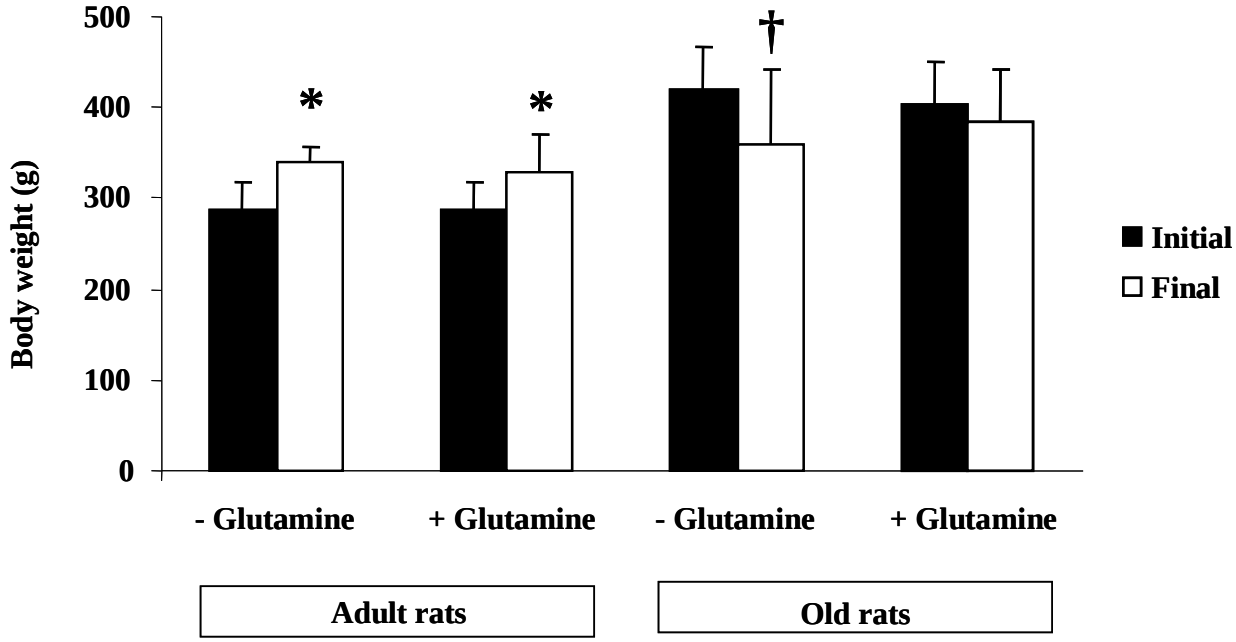
Figure 1. Effect of long term intermittent glutamine supplementation with treatment disruption before sacrifice on body weight of Wistar female rats. Initial body weight consisted in rat body weight before the treatment with Gln. Final body weight consisted in the body weight at the time of the sacrifice. ANOVA was performed to discriminate among effects of time (T) (beginning of supplementation or time of the sacrifice after supplementation), aging (A) and supplementation (S) and their interaction (A*T, A*S, T*S, A*T*S). Significant effects of A*S and A*T*S were observed ($P < 0.05$).***** $P<0.05$ vs '- initial adult rats, † $P<0.05$ vs '- glutamine' treated very old rats.

Figure 2. Proton-decoupled ^{13}C nuclear magnetic resonance (NMR) spectrum of a perchloric extract of incubated enterocytes from adult rats. Medium incubation contains ~ 17mM of [1-^{13}C] glutamine. Two regions are shown (A) 70 to 20 parts per million (ppm) and (B) 184 to 174 ppm where ppm is chemical shift relative to tetramethylsilane. Abreviations Gln and Glu respectively mean glutamine and glutamate and number associated with abreviations refers to carbon position that ^{13}C atoms occupy within that compound. Peaks labeled cycloleucine consist in peak used for standardization of areas under peaks after integration. X and Y are unknown peaks.

Figure 3. Glutamate production from [1-^{13}C] glutamine in incubated enterocytes from adult and very old rats treated or not with glutamine before sacrifice. Quantity of glutamate was calculated from the sum of areas of C2, C3 and C4. ANOVA was performed to

discriminate among effects of aging (A) and supplementation (S) and their interaction (A*S). Significant effects of A*S was observed ($P < 0.05$).***** $P<0.05$ vs all other groups

Figure 4. CO_2 production from [U-^{14}C] glutamine in incubated enterocytes from adult and very old rats treated or not with glutamine before sacrifice. ANOVA was performed to discriminate among effects of aging (A) and supplementation (S) and their interaction (A*S). Significant effects of A and S were observed ($P < 0.05$).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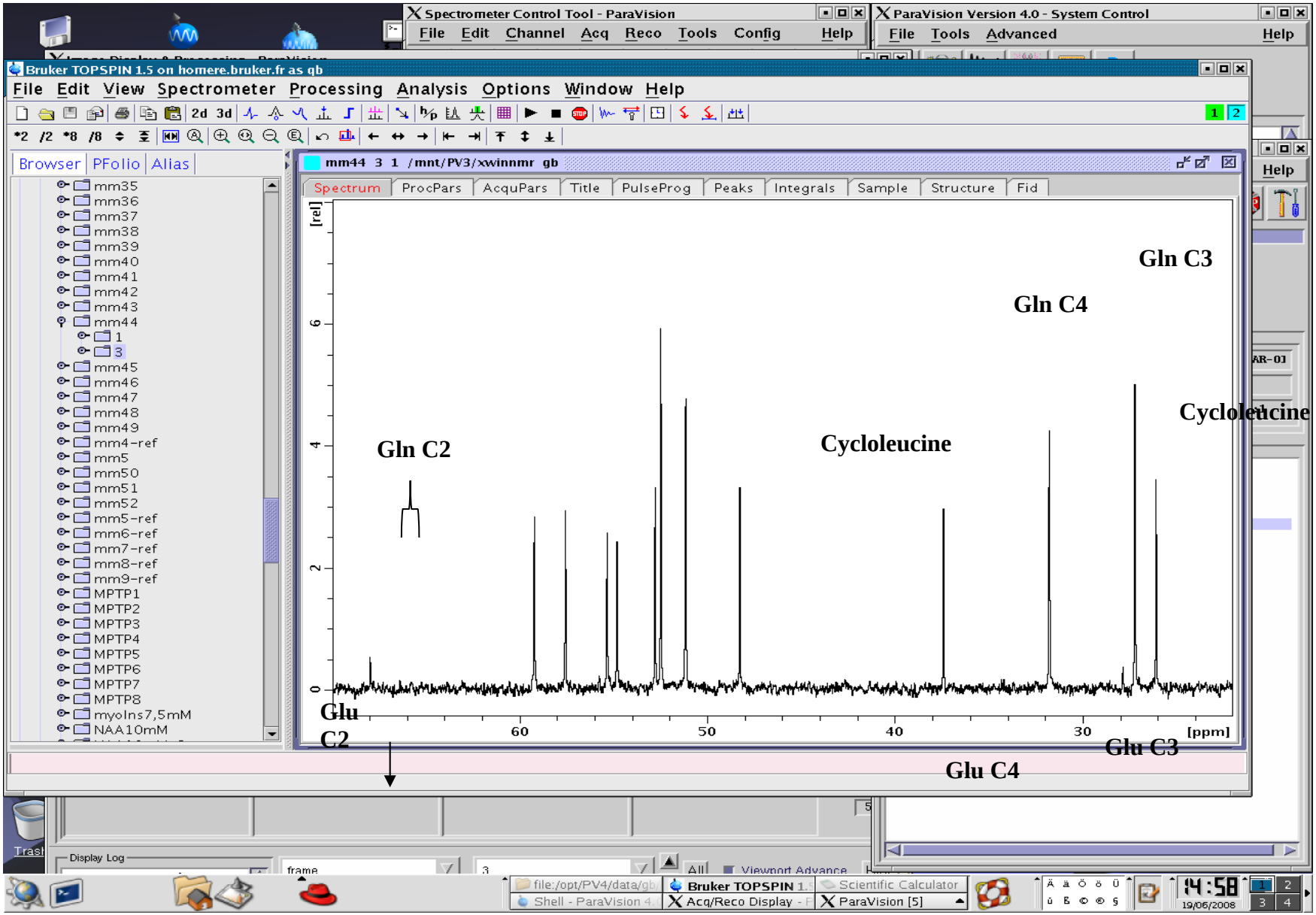
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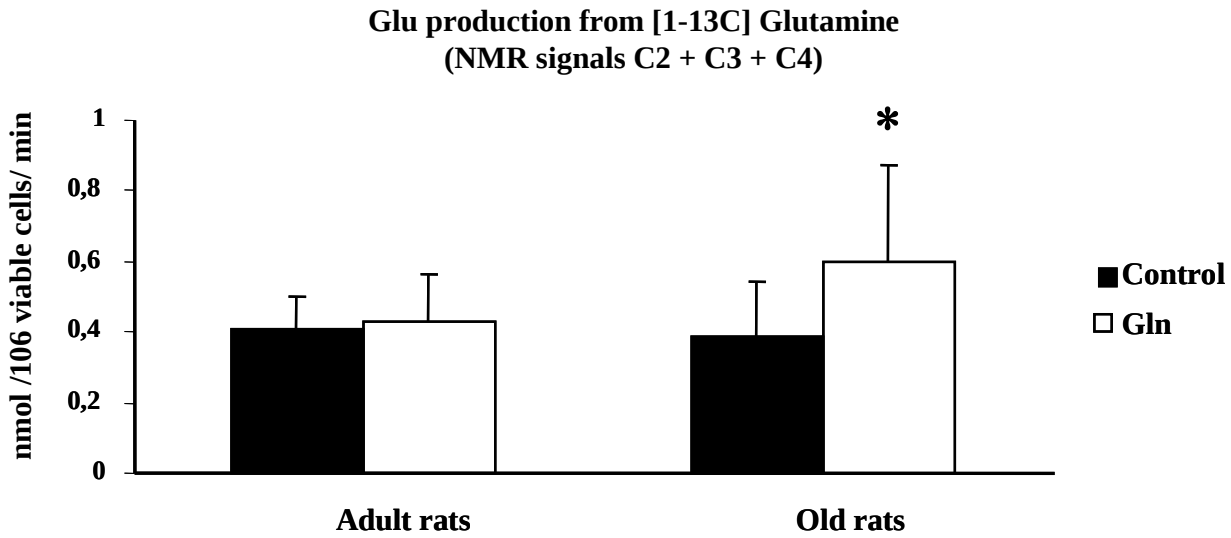
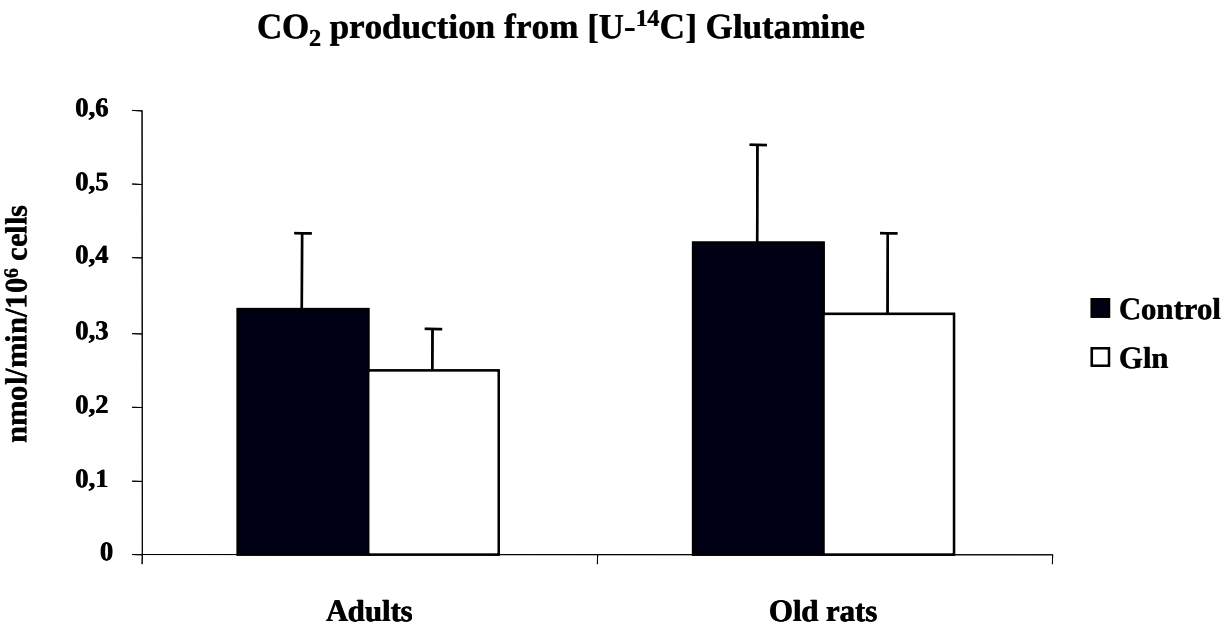


Effet age : non significatif
Effet traitement : significatif



Effet age : significatif
Effet traitement : significatif

Table 1 Effect of long term intermittent glutamine supplementation with treatment disruption 15 days before sacrifice on intestine histomorphometry in female Wistar rats.

	Villus height (µm)	Crypt depth (µm)	Villus height - Crypt depth (µm)
Adult rats			
Without glutamine	506±77	125±22	380±61
With glutamine	546±88	155±29	390±64
Very old rats			
Without glutamine	606±107	163±50	442±63
With glutamine	558±90	171±35	387±67
Significant effect of ANOVA	NS	A	NS

Values are means ± SD (n=10). ANOVA was performed to discriminate among effects of aging (A), supplementation (S) and their interaction (A*S). Significant effects of A was indicated ($P < 0.05$). NS: not significant.