# TO STUDY AMMONIA METABOLISM THROUGH ACTIVITIES OF GLUTAMINE SYNTHETASE (GS) AND PHOSPHATE-ACTIVATED GLUTAMINASE IN PIGS WITH ACUTE LIVER FAILURE

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# **CONTENTS**

# Abstract

*Background:* Acute liver failure (ALF) results in disturbed ammonia metabolism, urea synthesis capacity decreases and hyperammonemia develops (1). Ammonia accumulates to toxics levels in the brain, leading to cerebral oedema (2). Glutamine synthetase (GS) detoxifies (temporarily) ammonia to glutamine. However, glutamine is split by phosphate-activated glutaminase (PAG) again to ammonia and glutamate (1).

We aimed to investigate whether organ specific changes in both GS and PAG activities are involved in the development of hyperammonemia in ALF. We also studied these relations in the context of a novel ammonia lowering treatment concept, L-Ornithine + Phenylbutyrate (OP)(2). *Methods:* 24 female pigs were randomised into three equal groups: ALF (induced by liver devascularisation), ALF + OP and sham-operated. Arterial ammonia was measured every 2 hours. At the end of the experiment at T=8 hrs, the animals were sacrificed, tissue samples from the kidneys, lungs, muscles, duodenum, brain and ileum were dissected and "flash" frozen at -80 °C in liquid nitrogen for storage before further tissue processing.

To measure GS activity we applied a modified version of the methods previously described by Minet et al (3) and Seiler et al(4). PAG activity was determined by measuring ammonia production following incubation for one hour at 37 °C with O-phthaladelhyde (OPA) (5).

*Results:* GS activity in ALF group, in almost all tissues studied, was increased. PAG activity, in all tissues studied, was increased. We observed reduction in PAG activity in all tissues in ALF-OP treated animals. *Conclusion:* Increased GS activity in muscles, lungs and small intestines contributes to ammonia detoxification during ALF. Increased PAG activity in kidney, small intestines and lungs contribute to systemic hyperammonemia in ALF. OP treatments lead to decrease in PAG activity, hence reduction in arterial ammonia and prevention of intracranial hypertension in pigs with ALF.

# Background

The liver plays a central role in whole body nitrogen metabolism. Liver failure and resulting hepatocellular dysfunction results in disturbances in body nitrogen homeostasis (1). This is due to impaired hepatic urea synthesis capacity, intra- and extra hepatic portacaval shunts and because of  reduced perivenous glutamine synthesis capacity, resulting in reduced capacity to detoxify ammonia in the liver(1).

This leads to ammonia accumulating to toxics levels in the brain and result in increased ICP and hepatic encephalopathy (HE) (1). HE is a common, reversible neuropsychiatric complication of lever disease affecting about 20-30% patients with cirrhosis. HE may only affect quality of life (e.g. impairments in attention; coordination; driving ability), but in some patients this progresses to coma and death (2). In patients with ALF, an arterial ammonia level of >150 µmol/L has been found to be predictive of brain herniation (6). Hence, ammonia-lowering strategies remain the main therapeutic target for the treatment of HE. However, currently available treatment protocols have been shown to be largely ineffective (6, 7).

In liver failure the most important temporary alternative pathway for ammonia detoxification is the formation of glutamine from ammonia and glutamate catalysed by the enzyme glutamine synthetase(GS) (figure 1)(11).The glutamine formed is then taken up by other organs, (i.e. small intestine and kidney) and split by the intra-mitochondrial phosphate-dependent enzyme glutaminase (PAG) into glutamate and ammonia(1). Thus, ammonia is regenerated instead of being excreted through the kidneys, which causes an ammonia re-bound effect (9, 10).

Many ammonia-lowering strategies have been directed towards reducing the production of ammonia in the gut, such as with non-absorbable antibiotics and cathartics (non-absorbable disaccharides) (11). A recently published meta-analysis concluded that there was lack of evidence for the routine use of these strategies for the treatment of hepatic encephalopathy (HE) in patients with cirrhosis. No clinical trials have been performed with these strategies in patients with ALF (12, 13). Accordingly, treatment of hyperammonemia and intracranial hypertension in ALF remains an unmet clinical need.

One approach is to increase the amount of substrates to stimulate ammonia-removing pathways such as in the urea cycle and/or glutamine synthetase (GS). The administration of amino acids L-ornithine and L-aspartate to patients with cirrhosis as well as to rats with

portacaval anastomosis resulted in an increase in urea production (14, 15). Targeting GS by providing the substrate (glutamate) is potentially an effective way of detoxifying ammonia through the production of glutamine. However, as already mention above, instead of being excreted through the kidneys, glutamine is capable of being metabolised in the gut or kidney and regenerating ammonia, causing an ammonia-rebound effect (2). Therefore, the understanding of interorgan ammonia metabolism is the basis of the hypothesis in which the combination of L-ornithine and phenylacetate would increase excretion of ammonia in pigs with ALF (figure 2) (2). Phenylacetate is an established treatment of hyperammonemia in patients with urea cycle enzyme deficiencies (16-18). These patients have markedly increased blood glutamine concentrations and to prevent an ammonia-rebound effect, phenylacetate conjugates with glutamine producing phenylacetylglutamine which cannot be metabolised through glutamine synthetase and instead is excreted through the kidneys (16-18). In a recently performed study we aimed to test this hypothesis in a large animal (porcine) model of ALF (induced by hepatic devascularisation) which develops typical clinical and biochemical features of ALF (19-22). Our focus was to demonstrate the efficacy of L-ornithine phenylacetate (OP) and whether a reduction in plasma ammonia is associated with attenuation in both brain ammonia concentrations and intracranial pressure (ICP).

**Results from OP study**

The results showed that the administration of OP to pigs with ALF successfully reduces arterial and extracellular brain ammonia levels and as a result prevents any rise in ICP during the experimental period (figures 3-5).

**Aim for the present study**

The aim of this study was to investigate whether organ specific changes in both GS and PAG activities are involved in the pathogenesis of the development of hyperammonemia in liver failure. Furthermore, we aimed to study these relations in the context of this novel ammonia lowering treatment concept, OP.

## METHODS

### Study outline

The study was performed in the Surgical Research Laboratory at the University of Tromsø, Norway and was approved by the Norwegian Experimental Animal Board. A well-validated and characterised large animal model which recapitulates the cardinal features of human ALF including acute hyperammonemia and increased ICP was used (16). Twenty-four female pigs weighing 27-35 kg were randomised using the sealed envelope system into three groups:

    (i)      ALF + vehicle                               (n=8)

    (ii)     ALF + L-Ornithine + Phenylacetate       (n=8)

    (iii)    Sham operated + vehicle                   (n=8)

One pig (ALF + OP) was excluded due to surgical complications. Two pigs (one ALF and one sham pig) were excluded due to technical errors. Accordingly, data from 21 pigs was thus included in the present study.

At the end of the experiment at T=8hrs, immediately after the animals were sacrificed, tissue samples (2 cm x 2 cm) from the kidneys, lungs, liver, muscles (hindleg), duodenum and ileum were dissected and quickly "flash" frozen at -80 °C in liquid nitrogen for storage before further tissue processing.

### Glutamine synthetase activity assay

To measure glutamine synthetase activity we applied a modified version of the methods previously described by Minet et al.(3) and Seiler et al.(4). Tissue was homogenized with 10 x volume of the imidazole-EDTA buffer, and subsequently diluted using the same buffer. Reaction buffer was composed of the following: Imidazole (80.7mM), Sodium arsenate (40.3mM), Sodium ADP (81uM), L-Glutamine (30.3mM), $MnCl_2 4H_2O$ (1mM). The pH was adjusted to 7.0. Immediately before use aliquots were diluted 1:1 with 1 M NaOH and then adjusted to pH 7.0. This solution was further diluted 1:1 with water. Tissue homogenate (80 µl) was mixed with 70 µl reaction mixture. Hydroxylamine solution (2M; 10µl) was added immediately before the samples were incubated (usually for 20 minutes) at 37 °C under gentle shaking. The reaction was terminated by adding 80µl of a solution of 2.42% FeCl and 1.45% TCA in 1.82% HCl Insoluble material was removed by centrifugation and the

absorbance of the supernatant was measured at 540 nm. Blanks were made as the incubation mixtures, except that instead of the enzyme preparation (i.e. tissue homogenate) 80 µl of the imidazole-EDTA (homogenisation buffer) buffer were added. Glutamic acid-γ-hydroxamate was used as standard (range 0-10 mM). Appropriate dilutions were prepared as needed by adding water.

*Calculations;*

$$ADP; Mn^{2+}; AsO_4^{3+}$$

Glutamine + H$_2$NOH $\xrightarrow{\hspace{4cm}}$ Glutamyl-γ-hydroxamate + NH$_3$

$$GS$$
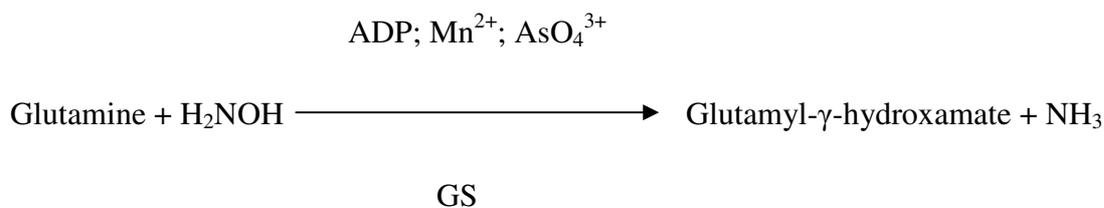
As shown in the formula, the relation is 1:1, which allowed us to calculate the enzyme activity directly from the concentrations in the standard curve.

**PAG activity assay**

Mitochondrial protein was measured by the determined method of Bradford with bovine serum albumin (BSA) as standard (Stigma). 3 tubes were prepared: sample, buffer and control sample, then 35 µl of reaction buffer (K$_2$HPO$_4$ 150 mM pH 8; L-Gln (L-Glutamine, Sigma, ref. 49419) 171 mM, NH4Cl 1 mM, adjust to pH 8) was added to 2 tubes (buffer tube and sample tube, not control sample one). After sample incubation in a wheel for 30 min at 4 ºC, 25µl of mitochondrial solution was added (in incubation buffer with protease inhibitor, Triton X-100, β-mercaptoethanol) to obtain protein concentration between 5 to 10 mg/ml. After incubation for 45 min at 37 degrees Celsius the reaction was stopped with 10 µl of 100 g/L trichloroacetic acid (TCA). Blanks were prepared separately following the incubation of the reaction medium/buffer and samples were mixed before TCA. When the sample-mixture reaction was stopped, the reaction mixture was placed in ice for 15 min and then centrifuged at 12, 000 r/min for 5 min at 4 ºC. The micro-titre plate was loaded with 5 µl of supernatant and 150µl of OPA reagent (0.2 mol/L K2HPO4, ph 7.4; 56 ml/L ethanol; 10 mmol/L O-Phthalaldehyde; 0.4 mmol/L β-mercaptoethanol) to each well. The plate was incubated in the dark at room temperature for 45 min. Standards for NH$_4$Cl were prepared to concentrations of 50-300mg/L. Absorbance was measured at 405 nm with a spectrophotometer.

*Calculation;*

Total Volume x Dilution in plate

——————————————————

Time x Volumen de muestra

$$\frac{70 \times 10^{-6} \times 155/5}{60 \times 25 \times 10\text{-}3} \quad X\ 1000 = mU/ml$$

Specific activities (SA) of enzymes were expressed in international units per milligram of mitochondrial or homogenate protein (samples and tubes of standard curve too) (5).

**Statistical analysis**

Data was expressed as mean ± SEM. Statistical analysis were performed using the Statistical Package for the Social Science, version 14.0 for windows (SPSS, Chicago, Ill). The Mann-Whitney U test was used to test for differences between groups. Probability values $P \leq 0.05$ were considered significant for all tests applied.

# RESULTS

**GS activity**

*Muscle Glutamine Synthetase Activity*: Hindleg muscle glutamine synthetase (GS) activity was higher in the ALF+OP (40.6±21.1 nu/mg protein) and the ALF groups (19.1±9.9 nu/mg protein) compared with the sham operated groups (8.9±1.3 nu/mg protein) but this difference did not reach statistical significance (P=0.10). (Figure 6)

Kidney GS activity: Kidney GS activity was higher in ALF+OP (290.27 ±38.85nu/mg protein) and the SHAM groups (237.51 ±13.65) compared with the ALF operated groups (236.00±15.92), (figure 7). The difference between SHAM and ALF is not significant statistically (p>0.05).

*Lung Glutamine Synthetase Activity*: Lung glutamine synthetase (GS) activity was higher in the ALF (115.76±16.09 nu/mg protein) compared with the SHAM groups (107.52±15.78nu/mg protein), and compared with the ALF+OP operated groups (67.58±5.94nu/mg protein). The difference between ALF and SHAM did not reach statistical significance. (Figure 8).

*Duodenal Glutamine Synthetase Activity*: Duodenal glutamine synthetase (GS) activity was higher in ALF groups (100.33± 18.03 nu/mg protein) and the ALF +OP groups (79.46 ± 10.47 nu/mg protein) compared with the sham operated groups (76.35±7.38nu/mg protein).(Figure 9).

*Ileum Glutamine Synthetase Activity*: Ileum glutamine synthetase (GS) activity was higher in the ALF (138.46±16.54nu/mg protein) compared with the SHAM groups (97.11± 15.50nu/mg protein) and with the ALF+OP operated groups (92.09±10.97nu/mg protein) (figure 10). The difference in GS activity in ALF and sham is significant statistically (p>0, 05).

**PAG activity**

*(Muscle PAG activity*:   Results not available)

 *Kidney PAG activity*: Kidney PAG activity was higher in the ALF (73.00±10.75 nu/mg) compared with the ALF+OP groups (67.07±8.60 nu/mg protein) and with the sham operated groups (52.96±13. 19nu/mg protein). PAG activity in kidneys of ALF group is significantly higher than in SHAM operated group (p>0.05). (Figure 11).

*Lung PAG activity*: Lung PAG activity was higher in the ALF (106.67±11.00 nu/mg protein) compared with the ALF+OP groups (107.86±10.29 nu/mg protein) and with the sham operated groups (89.04±8.69nu/mg protein). PAG activity in lungs

of ALF group is significantly higher than in SHAM operated group (p>0.05). (Figure 12).

*Duodenum PAG Activity*: Duodenum PAG activity was higher in the ALF (303.70±62.27 nu/mg protein) compared with the ALF+OP groups (295.76±43.17nu/mg protein) and with the sham operated groups (197.38±43.78 nu/mg protein). (Figure 13).

*Ileum PAG Activity*: Ileum PAG activity was higher in the ALF (193.37±32.25 nu/mg protein) and the ALF+OP groups (133.62±43.47 nu/mg protein) compared with the sham operated groups (109.62±18.21 nu/mg protein).(Figure 14)

## DISCUSSION

The liver plays a central role in whole body nitrogen metabolism. Acute liver failure results in disturbed body nitrogen homeostasis due to impaired hepatic urea synthesis capacity (1) and the intra- and or extrahepatic shunting of portal blood into systemic circulation. This leads to an alteration in interorgan ammonia trafficking and hyperammonemia, which contributes to the risk of development of cerebral oedema (2). This is in connection with the toxicity of ammonia at elevated levels in the body. In this situation, the most important temporary alternative pathway for ammonia detoxification is the formation of glutamine from ammonia and glutamate catalysed by enzyme glutamine synthetase (8). Other organs in the body can take up glutamine, where it is split by the intra-mitochondrial phosphate –dependent enzyme glutaminase into glutamine and ammonia (1). Thus, ammonia is regenerated instead of being excreted through the kidneys, and causing ammonia re-bound effect (9, 10).

The aim of our study was to investigate whether organ specific changes in both GS and PAG activities are involved in the pathogenesis of the development of hyperammonemia in liver failure. Furthermore, we aimed to study these relations in the context of this novel ammonia lowering treatment concept, OP.

Muscle GS activity is higher in ALF group compared to sham operated group. In ALF-OP treated there was even further increase GS activity. This data support data from a study carried out early which showed that skeletal muscle metabolism plays an important role in ammonia metabolism in chronic and acute liver failure. Skeletal muscle constitutes quantitavely most important localization of the enzyme glutamine synthetase (1).

Our data show a slight reduction in the activity of GS in the kidneys of ALF group compared with sham groups. In ALF-OP treated group, there is increase in GS activity. This data is difficult to interpret.

Our data shows a trend towards an increase in the lung GS activity in the ALF groups compared to sham animals. In ALF-OP treated group, we observed reduction in GS activity in the lungs. Data from previous studies shows that lungs are able to remove ammonia from circulation during the initial stage of ALF (24).

In the Duodenum, our data shows also a trend towards increase in GS activity in the ALF group compared with sham groups. In ALF-OP treatment group there is reduction in GS activity.

GS activity was increased in the ileum of ALF groups compared to that of sham operated groups. In the ALF-OP treatment group we noticed reduction in GS activity.


Data for muscle PAG activity was difficult to interpret, so it was not included under results. However, our data shows that PAG activity is increased in the kidneys of ALF group compared to sham operated groups. This support a study carried out on PCA rats which showed increased PAG activity in kidneys. It was also concluded in that study that this could contribute to hyperammonaemia (25). In ALF-OP treated groups, there is reduction in PAG activity.

PAG activity was increased in the lungs of ALF pigs than in sham operated groups. In ALF-OP treated group, there was a slight increase in PAG activity.

In the Duodenum, PAG activity was increased in the ALF group than in sham operated groups. A study has shown that increased PAG activity in the duodenum contributes significantly to systemic hyperammonaemia (25). In ALF-OP treated group we observed slight reduction in PAG activity.

PAG activity was increased in the ileum of ALF groups compared to that of sham operated groups. This is in agreement with previous studies in animal models and patients with liver disease have pointed to an important role for the gut and kidneys in the production of

ammonia (25). This means that ileum plays a role in contributing to systemic hyperammonaemia during ALF, in addition to duodenum and kidneys, major contributors to systemic hyperammonaemia. In the ALF-OP treatment group we noticed reduction in PAG activity in the ileum.

The data with respect to GS and PAG activity in the brain is difficult to interpret due to large variations. Therefore the data was not included under results.

Overall, it is possible that during the measurement of absorbance, high background noise could have affected the results of GS and PAG activities. Poor mixing of test materials or of different solutions could also have affected results. Results of GS and PAG activities could also have been affected by titration mistakes.

In conclusion, the results of our study shows a strong trend to increase in GS activity in ALF group, in almost all tissues studied. This could contribute to the temporary alternative pathway for ammonia detoxification, by formation of glutamine from ammonia and glutamate. Our result also shows a very strong trend towards increase in PAG activity, in all tissues studied. This could contributes to the so called ammonia re-bound effect, where other organs in the body, most especially kidney and gut, take up glutamine split it with the help of PAG enzyme into glutamine and ammonia. Thus, ammonia is regenerated instead of being excreted through the kidneys. This contributes to systemic hyperammonaemia during ALF, which leads to cerebral oedema.

Our data also indicate that OP can stimulate glutamine production through GS solely by increasing its substrate (glutamate). We also observed a strong tendency towards reduction in PAG activity in all tissues in ALF-OP treated animals. This is because phenylacetate, in OP, conjugate with ornithine-derived glutamine, which is substrate for PAG, forming phenylacetylglutamine which is excreted into the urine. This lead to reduction in substrate for PAG, thus decrease in PAG activity in ALF-OP group. Therefore, OP successfully attenuated an increase in arterial ammonia which was accompanied with a decrease in cerebral ammonia and prevention of intracranial hypertension in pigs with ALF.

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## Legends to figures

<u>Figure 1</u>

Schematic illustrating that glutamine is an intermediate ammonia (NH3) sink. (a)Interaction of glutamate and ammonia by glutamine synthetase (GS) produces glutamine, whereas (b) the interaction of glutamine with glutaminase produces glutamate and ammonia (2).

<u>Figure 2</u>

Shows that a combination of L-ornithine and phenylacetate (OP) can potentially reduce toxic levels of ammonia, in that L-ornithine increasing glutamine production (ammonia removal) through glutamine synthetase in skeletal muscle and phenylacetate conjugating with this ornithine-derived glutamine, forms phenylacetylglutamine which is excreted into the urine.

<u>Figure 3-5</u>

Shows that the administration of OP to pigs with ALF successfully reduces arterial and extracellular brain ammonia levels and as a result prevents any rise in ICP during the experimental period.

<u>Figure 6</u>

GS activity in hindleg skeletal muscle from pigs with ALF, ALF+OP, and sham operated controls Mean+SEM.

<u>Figure 7</u>

GS activity in kidneys from pigs with ALF, ALF+OP, and sham operated controls. Mean+SEM.

<u>Figure 8</u>

GS activity in lungs from pigs with ALF, ALF+OP, and sham operated controls. Mean+SEM.

Figure 9

GS activity in duodenum from pigs with ALF, ALF+OP, and sham operated controls. Mean+SEM.

Figure 10

GS activity in ileum from pigs with ALF, ALF+OP, and sham operated controls. Mean+SEM.

Figure 11

PAG activity in kidney from pigs with ALF, ALF+OP and sham operated controls. Mean+SEM

Figure 12

PAG activity in lungs from pigs with ALF, ALF+OP and sham operated controls. Mean+SEM

Figure 13

PAG activity in duodenum from pigs with ALF, ALF+OP and sham operated controls. Mean+SEM

Figure 14

PAG activity in ileum from pigs with ALF, ALF+OP and sham operated controls. Mean+SEM

# FIGURES1-14

Figure 1
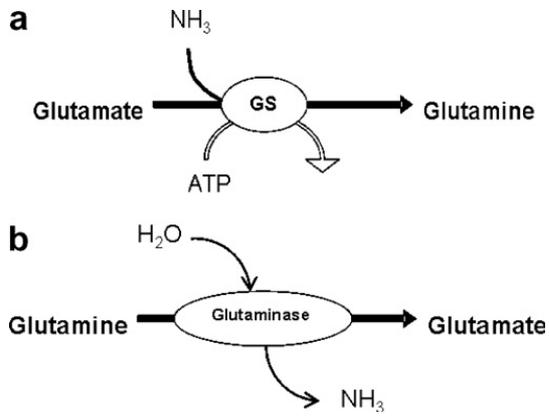


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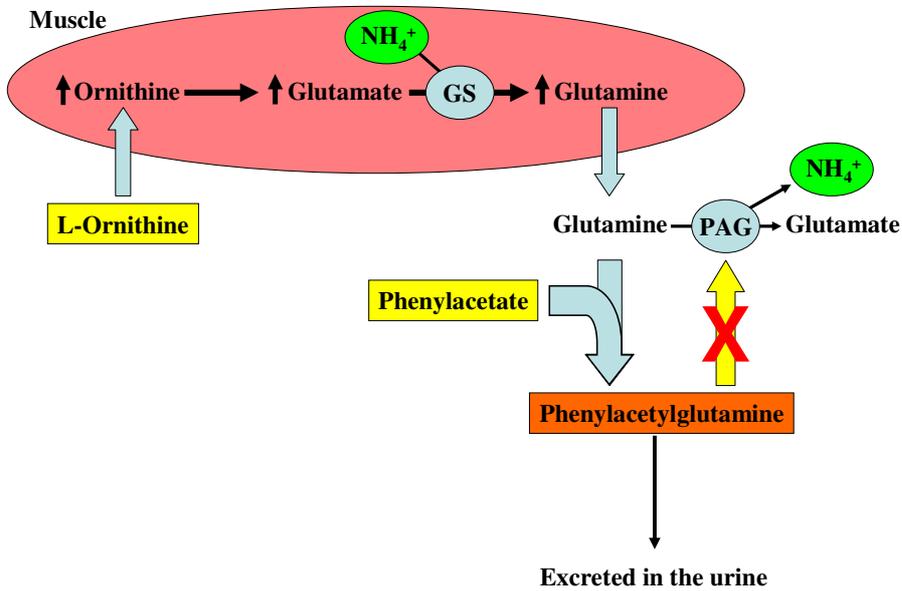
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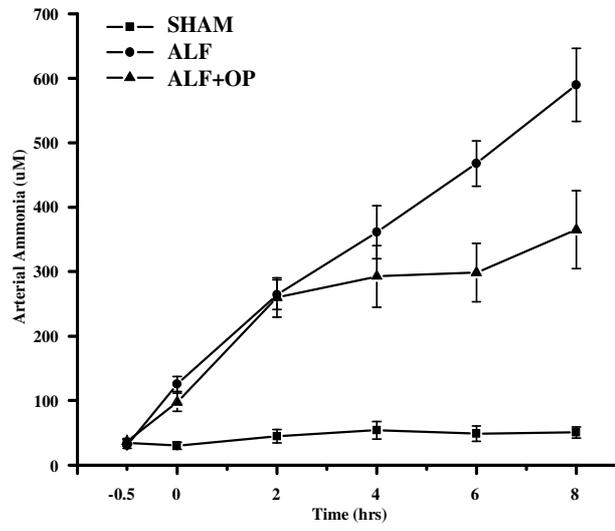


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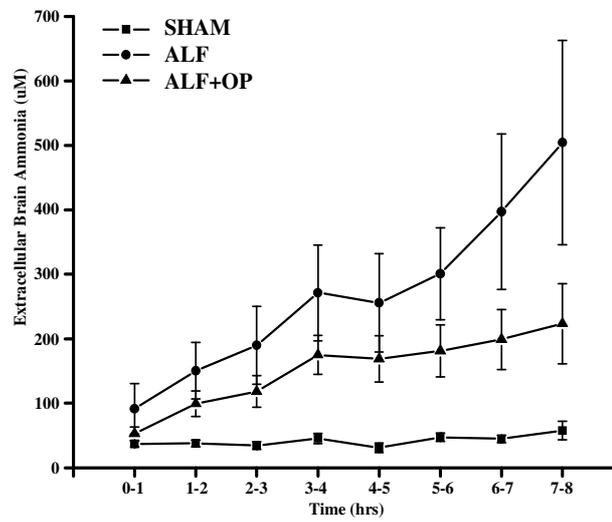
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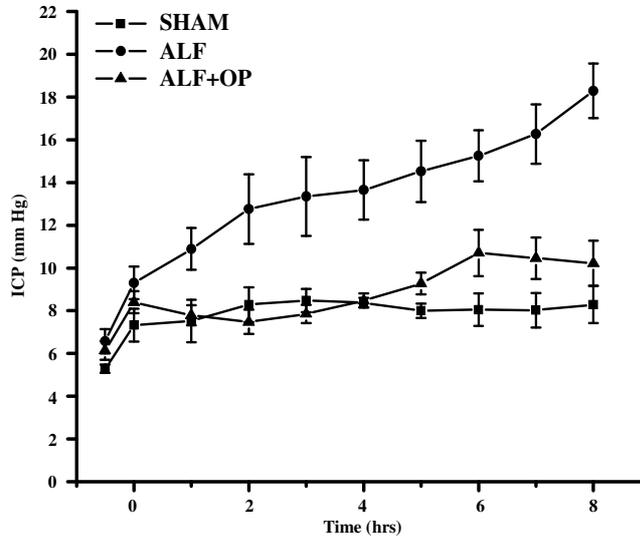


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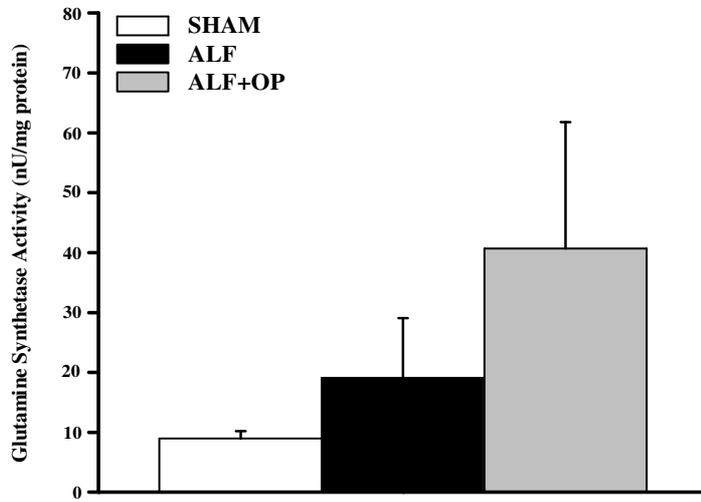
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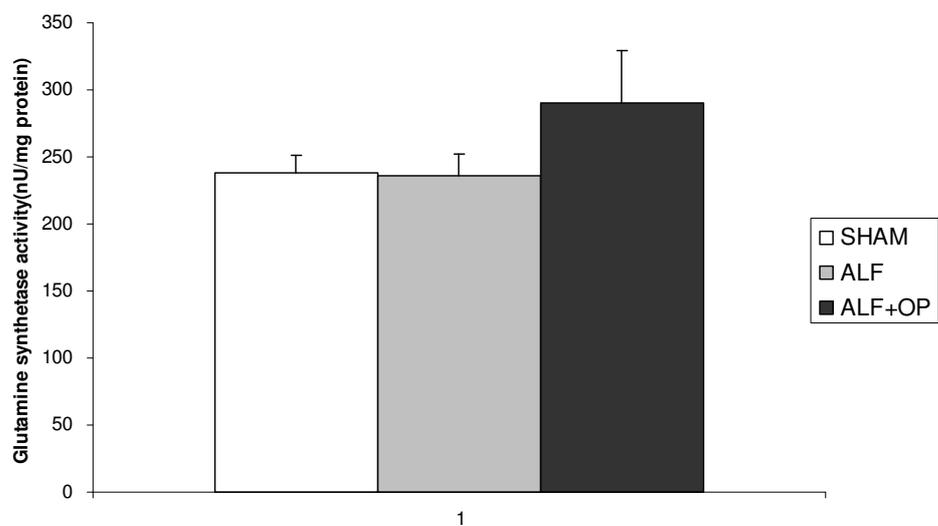


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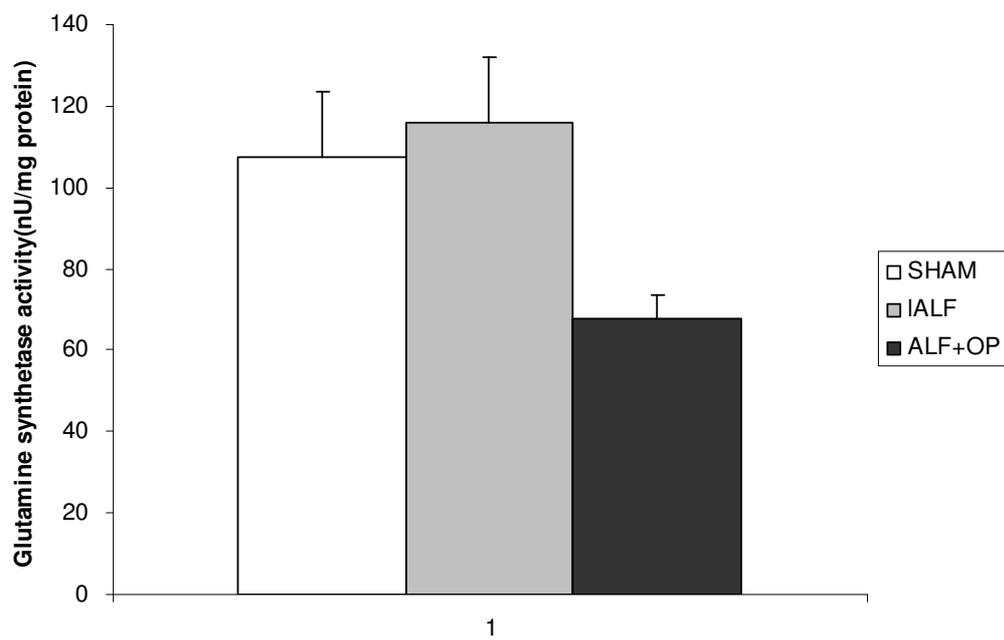
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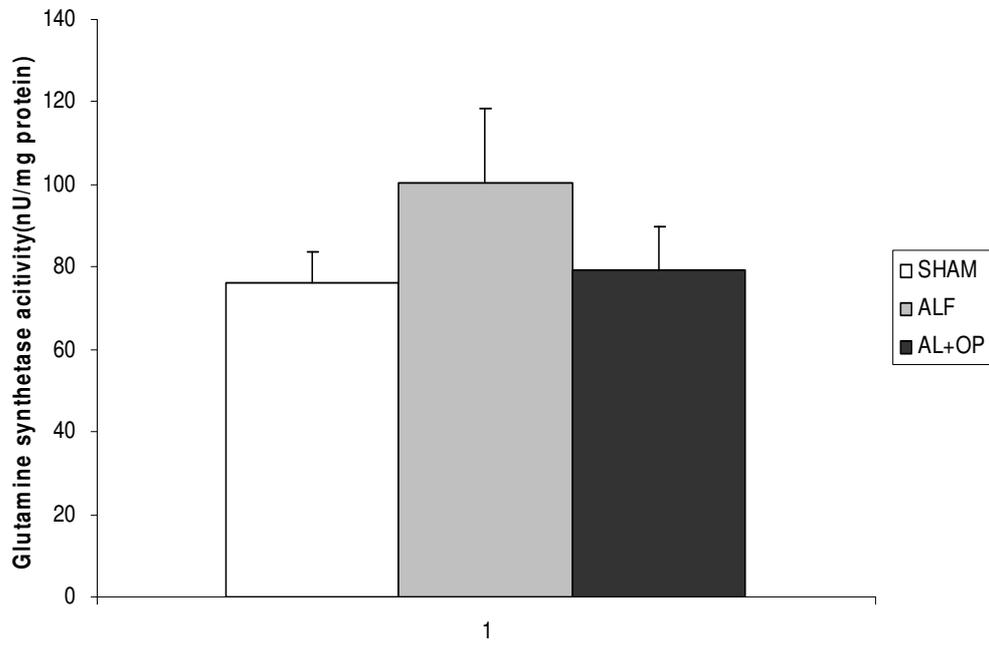


Figure 9

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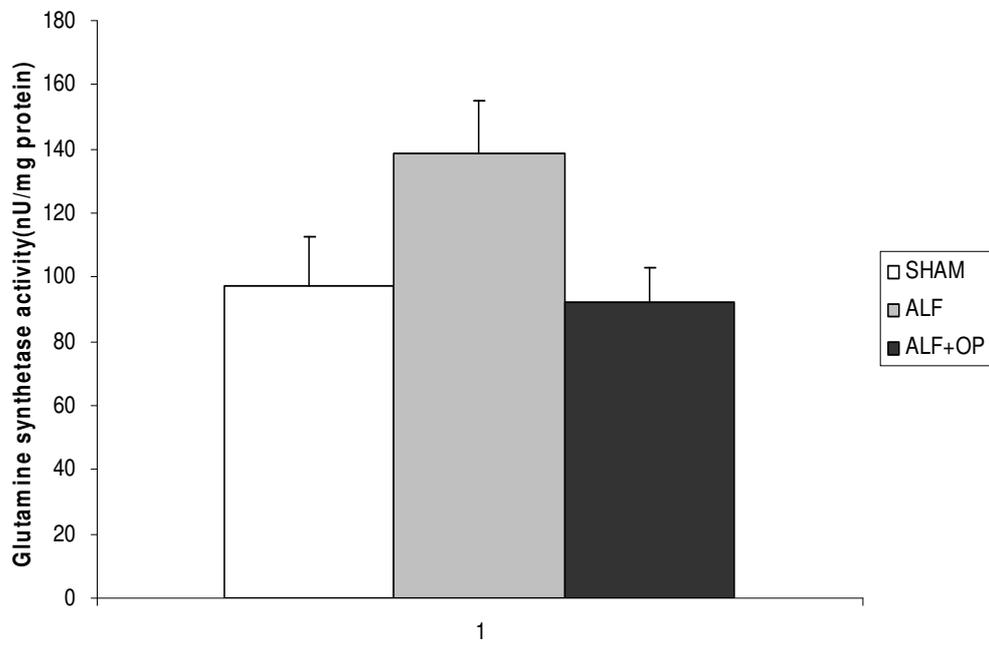


Figure 10

Figure 11
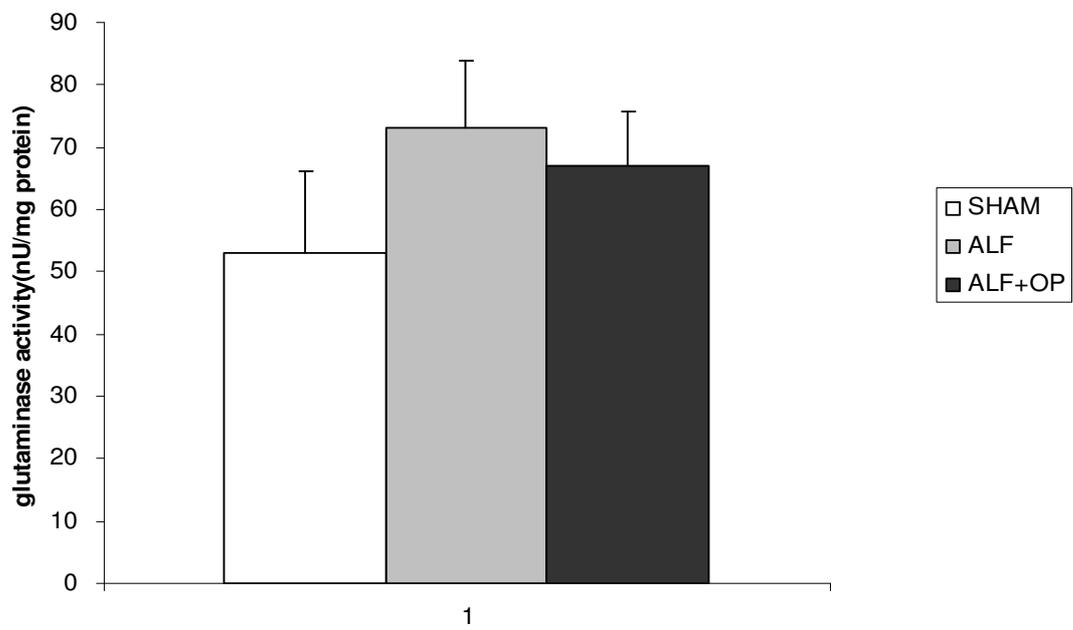


Figure 11



Figure 12

Figure 13



Figure 14