

# Hyperammonemia induced oxidative stress in cirrhotic rats without promoting differential protein expression in the brain cortex: A 2D-DIGE analysis\*

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## ABSTRACT

Oxidative stress induced by a high ammonia concentration could modify protein expression in the brain. This study was undertaken in order to investigate the impact of hyperammonemia, caused by thioacetamide (TAA) in rats, on brain cortex protein expression using 2D-DIGE, and analyzing its role in the pathogenesis of HE. Hyperammonemia was induced with TAA. Ammonia and active oxidants were measured by L-glutamate dehydrogenase and dichlorodihydrofluorescein diacetate methods, respectively. Lipid peroxidation and protein oxidation biomarkers were also studied. Differential protein expression in the cortex of TAA- and control-rats was studied by 2D-DIGE. Image analysis was performed using the DeCyder™ Software. Ammonia concentration in plasma and brain tissue was higher in TAA-rats compared to control-rats, 3.12 and 2.43 fold higher, respectively. Active oxidants production in TAA-rats was increased by 2.7 fold compared to control-rats. Measurements of MDA, HNE and carbonyl groups, biomarkers of lipid peroxidation and protein oxidation respectively, were found to be statistically significantly increased in TAA-rats compared to control-rats (3.16-, 2.44- and 1.95-fold, respectively), reflecting the presence of oxidative stress in the brain of TAA-rats. 2D-DIGE analysis of brain cortex protein allowed the detection of 2896 spots, however, image analysis showed no statistically significant differential protein expression between the proteins expressed in TAA- and control-rats. No statistical significant differential protein expression in the cortex of TAA-rats was observed, although oxidative stress biomarkers for lipid and proteins were higher in the brain of TAA-rats than in

control-rats. These results support the idea that oxidative post-translational modifications are implicated in HE physiopathology.

**Keywords:** Oxidative Stress; Hyperammonemia; Cirrhosis; Hepatic Encephalopathy; Protein Expression

## 1. INTRODUCTION

Hepatic encephalopathy (HE) is the major neurologic disorder occurring in patients with severe liver disease which presents in chronic and acute forms. Chronic HE is a neuropsychiatric disorder which commonly occurs in the setting of alcoholic cirrhosis and is often associated with changes in personality, altered mood, decline in intellectual capacity and abnormal muscle tone. Acute HE (acute liver failure) has a high mortality rate (80% - 90%) due to the development of brain edema and increased intracranial pressure, and often presents with the abrupt onset of delirium, seizures and coma [1]. Although the pathophysiology of HE remains unclear, it is well established that ammonia plays a major role [2,3]. Ammonia accumulates in the brain in both acute and chronic liver failure, leading to a series of metabolic, energetic and signal transduction-related alterations [4]. However, it appears that other factors may also contribute. In particular, microglial activation and brain inflammation correlate with the progression of encephalopathy and the onset of brain edema in rats with acute liver failure [5]. Moreover, increased expression of the endothelial and inducible isoforms of nitric oxide synthase as well as heme oxygenase-1 [6] and direct oxidative biomarkers [7,8] have been reported in animal models, suggesting that oxidative/nitrosative stress also plays a significant role in the pathogenesis of brain complications observed in liver failure, and particularly in HE.

All these data indicate that oxidative stress is impli-

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cated in the development of HE, but they are not able to give any precise information about the mechanisms and proteins/enzymes involved in the physiopathology of HE. Therefore, the aim of the present work was to study the effect of oxidative stress, induced by high ammonia concentration, on rat brain proteins, using an animal model of HE. As a model of chronic HE, TAA-rats are often used because they allow studying longer exposition to high ammonia concentrations than the PCS-rat model [9]. In an attempt to gain knowledge on the pathogenesis of chronic HE, differential proteins expression was studied using a two dimensional differential in gel electrophoresis (2D-DIGE) approach, which allowed its identification and to study its relation with HE.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Chemicals, proteases, and antibodies used in these studies were mainly purchased from Sigma-Aldrich (Madrid, Spain). Electrophoresis and DIGE materials were purchased from GE Healthcare (Barcelona, Spain). Super-Signal West Pico Chemiluminiscent Substrate was purchased from Thermo Scientific Inc. (Rockford, USA).

### 2.2. Animals

Male Wistar rats ( $n = 20$ ) weighing 230 - 260 g at the beginning of the experiments, were used in the present study. Animals were supplied by the Centre of Production and Experimental animal of Espartinas, University of Seville, Spain. The animals were housed four per cage, and maintained at constant room temperature ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ), with artificial light-dark cycle of 12 h and a relative humidity of 65% - 70%, and free access to food and water during 2 weeks for acclimation. All animal procedures were performed with approval from the Sevilla University Animal Ethics Committees, under the guidelines of the directive 86/609/CEE of the European Community.

### 2.3. Animal Model of HE (Thioacetamide Treatment)

After the 2 weeks of acclimation, the animals were divided into two groups, and two animals per cage: control group ( $n = 10$ ) and thioacetamide (TAA) treated group (TAA-rats,  $n = 10$ ). The control-rats received food and normal water and served as a control for 16 weeks. The TAA-rats received food and continuous administration of 0.03% TAA (Sigma, Madrid, Spain) in the drinking water as an initial concentration that was subsequently modified according to weight changes in response to TAA during the induction of cirrhosis. The treatment was maintained during 12 weeks [10]. At the 12<sup>th</sup> week the

TAA-rats group had 8 animals ( $n = 8$ ) and the control-rat group had 10 animals ( $n = 10$ ). Animals were weighed weekly. Animals were killed by an overdose of pentobarbital and brains rapidly removed. Different brain *loci* (cortex, cerebellum, hippocampus) were dissected, frozen with liquid  $\text{N}_2$ , and stored at  $-80^{\circ}\text{C}$  until use. In this study the cortex was used.

### 2.4. Sample Preparation for Analytical Procedures

Brain samples (cortex) (100 mg) were homogenized in 1 ml homogenization buffer (10 mM Hepes, 137 mM NaCl, 4.6 mM KCl, 1.1 mM  $\text{KH}_2\text{PO}_4$ , 0.6 mM  $\text{MgSO}_4$ , 0.5  $\mu\text{g/ml}$  leupeptin, 0.7  $\mu\text{g/ml}$  pepstatin, 0.5  $\mu\text{g/ml}$  type II S soybean trypsin inhibitor, 40  $\mu\text{g/ml}$  PMSF), using a manual glass homogenizer (~50 passes). Homogenates were vortexed and sonicated for 10 s at 20% power in a sonication bath (Braun, Berlin, Germany), and centrifuge at  $2500 \times g$  in a microfuge (Mikro 200R, Hettich, Tübingen, Germany) to remove intact cells and nuclei. Protein concentrations were determined according to the Bradford method [11].

### 2.5. Measurement of Ammonia Concentration

Ammonia concentrations were measured in arterial plasma and brain tissue using the L-glutamate dehydrogenase method [12]. Plasma concentrations of ammonia were measured before the TAA-treatment and at the time of killing. Ammonia concentration was calculated and expressed as  $\mu\text{mol/l}$ . Brain tissue was homogenized as described above, and deproteinized in 5 volumes of ice-cold trichloroacetic acid (TCA) (100 g/l), and kept on ice for 15 min. After centrifugation at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , the supernatant was collected, neutralized with 2 M  $\text{KHCO}_3$  and centrifuged at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The neutralized supernatant was used to measure ammonia by the glutamate dehydrogenase method as described above.

### 2.6. Measurement of Active Oxidants Production

Brain active oxidants production was measured in brain homogenates obtained as described above by the Thorburne and Juurlink method [13] using 5-(and-6)-carboxy-20,70-dichlorodihydrofluorescein diacetate (DCFDA), with minor modifications [14].

### 2.7. Measurement of Low Molecular Dialdehydes

Malondialdehyde (MDA) was determined by HPLC according to Mateos *et al.* [15] as described in [8]. 4-Hydroxy-2-noneal (HNE) was determined according to standard immunodetection procedures [16] as described in [8].

## 2.8. Measurement of Carbonyl Groups

The total protein carbonyl levels in homogenates from brain tissue (cortex) obtained in homogenization buffer, as described above, of TAA- and control-rats were assayed following the Evans method [17] as described in [8].

## 2.9. Sample Preparation for Electrophoretic Separations

Cortex samples (in control or TAA-treated rats) were made powder with liquid N<sub>2</sub> and kept at -80°C. Approximately 10 mg of powder were mixed with 100 µl of lysis buffer (30 mM Tris-Cl pH 8.5, 2 M Thiourea, 7 M Urea, 4% (w/v) Chap). The homogenate were left on ice 10 minutes and sonicated 5 min. The homogenates were centrifuged in QIAshredder (Qiagen, Hilden, Germany) for eliminate the DNA and then centrifuged 15 min at 18,000 g at 4°C in a microfuge (Mikro 200 R, Hettich, Tübingen, Germany). The supernatant was taken and protein content was determined according to the method of Bradford [11].

## 2.10. Conventional 2D-Electrophoresis

Protein samples (150 µg) were loaded onto IPG strips (pH 4 - 7, 13 cm) previously hydrated over-night with rehydration buffer (2 M Thiourea, 7 M Urea, 4% (w/v) Chaps, 0.2% IPG Buffer pH 3 - 10, 0.2% (w/v)). The first dimension was performed using IPGphor 3 IEF system (GE Healthcare, Buckinghamshire, UK) at 20°C. The conditions for the IEF were performed according to the manufacturer's instructions (300 V during 3 hours, gradient to 1000 V during 3 h, gradient to 8000 V during 6 h, 8000 V during 6 h). The focused strips were equilibrated in equilibration buffer (50 mM Tris-Cl, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) dithiothreitol (DTT) for 15 min and transferred to 2.5% (w/v) iodoacetamide for 15 min. The SDS-polyacrilamide gel electrophoresis (SDS-PAGE) was run on 12% T and 2.7% C, 20°C, 450 V, 10 W, 15 mA/gel 10 min, 25 mA/gel 2 h using a Criterion Cell unit (Bio-Rad Laboratories Inc., Munich, Germany). Two gels were stained with SYPRO-Ruby protein gel staining to visualize total proteins and for protein identification.

## 2.11. Differential in Gel Electrophoresis

### 2.11.1. Sample Preparation and Protein Labeling with Cydye

Proteins samples (100 mg cortex) of control and TAA-treated rats were treated with 2-D Clean-Up Kit (GE Healthcare) following the manufacturer's instructions. The protein pellet was resuspended in lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS

pH 8) and adjusted to pH 8. Protein concentration was determined using the Bradford method. Prior to labeling the proteins with saturation dye, the amounts of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Invitrogen, USA) and dye were optimized according to the manufacturer's protocol.

Traditional three-dye system was employed to reduce variability associated with the multiple samples being compared [18]. Control and TAA-treated rat samples were labeled with N-hydroxy succinimidyl ester derivatives of the cyanine dyes Cy2, Cy3, and Cy5 following a standard protocol [19].

### 2.11.2. 2-D Gel Electrophoresis

Immobilised 24 cm linear pH gradient (IPG) strips, pH 3-11NL, were rehydrated in rehydration buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 0.5% IPG Buffer, 50 mM DTT) overnight, according to the standard guidelines. Iso-Electric-Focusing (IEF) was performed using an IPGphor™ 3 apparatus (GE Healthcare) for a total of 40 kV/hour at 20°C. Equilibrated IPG strips were transferred onto 12.5% uniform polyacrylamide gels poured between low fluorescence glass plates. Strips were overlaid with 0.5% w/v low-melting-point agarose in running buffer containing bromphenol blue. Gels were run using the Ettan Dalt 12 device (GE Healthcare) at 2.5 W/gel for 30 minutes then 100 W in total at 10°C until the dye front had run off the bottom of the gels.

### 2.11.3. Image Acquisition and Statistical Analysis

After second-dimension SDS-PAGE, gels were scanned using the Typhoon 9400 variable mode imager (GE Healthcare). Cy2 protein maps were scanned using a 488-nm laser with a 530-nm emission filter; Cy3 was scanned using a 532-nm laser with a 605-nm emission filter; Cy5 was scanned using a 635-nm laser with a 695-nm emission filter. Gels were scanned at 100 µm resolution. Image analysis was performed using the DeCyder™ Software version 7.0 (GE Healthcare). Gel-to-gel matching of the standard protein maps from each gel, followed by statistical analysis of differential protein abundance between samples, was determined using the biological variation analysis module (BVA) of DeCyder™. Analysis of variance (ANOVA) was applied to matched spots and the data were filtered to retain protein spots with  $p \leq 0.05$  determined by 1-way ANOVA and a fold-change of  $\geq 1.2$ . Using the EDA Version 1.0 (extended data analysis) module within the DeCyder software (GE Healthcare), data analysis was performed using principal components analysis (PCA) on proteins of interest [20].

## 2.12. Statistical Analysis

Results are expressed as mean  $\pm$  SEM. Statistical analy-

sis was carried out using Student's t test. A value of  $p < 0.05$  was considered significant.

### 3. RESULTS

In this work we have studied the effect of oxidative stress induced by hyperammonemia in an animal model (TAA-rats) of HE. Our aim has been the analysis of protein expression in the brain of TAA-rats and control-rats, and the correlation between these alterations and the pathophysiology of HE.

First of all, we have confirmed that the animal model used, TAA-rats, was a suitable model to study HE-associated hyperammonemia. Rats treated with TAA showed symptoms of brain injury such as lethargy, lack of spontaneous movement, loss of righting reflex, lack of response to pain; and as shown in **Table 1**, the level of ammonia in plasma ( $303.98 \pm 13.56 \mu\text{mol/l}$  versus  $97.50 \pm 9.23 \mu\text{mol/l}$ ) and in brain cortex ( $8.39 \pm 1.07 \mu\text{mol/g}$  tissue versus  $3.44 \pm 0.56 \mu\text{mol/g}$  tissue) were higher in TAA-rats compared with control-rats.

#### 3.1. Oxidative Stress Biomarkers

Once it was established that TAA-rats were hyperammonemic, and that its behavior was related with some of the hallmarks of HE and taken into account that oxidative stress has been implicated as possible cause of HE [7-9,14], we tested the hypothesis that high ammonia concentration could promote oxidative stress in the brain of TAA-rats. For this purpose the production of active oxidants (see **Figure 1**) and biomarkers of lipid and protein oxidations (see **Table 1**) have been quantified.

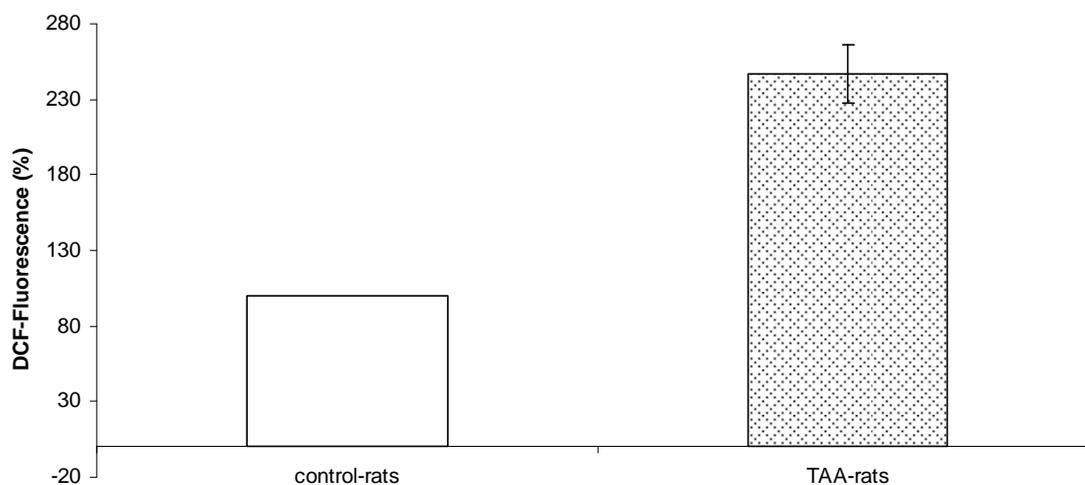
Measurements of global active oxidants production were carried out in a heterogeneous mixture of neurons, glial and microglial cells from TAA- and *sham*-rat brain using DCFDA as a probe. As **Figure 1** shows, active

oxidants production in TAA-rats was 2.47 times higher than that of the control-rats ( $247\% \pm 16\%$  versus  $100\%$ , respectively). Since DCFDA method mainly measured reactive oxygen species (ROS) we also investigated the production of reactive nitrogen species using nitric oxide (NO) as an appropriate marker of reactive nitric species (RNS). The production of NO was significantly higher in TAA-rats than in control-rats ( $21.80 \pm 1.57 \mu\text{mol/mg}$  protein versus  $14.76 \pm 1.26 \mu\text{mol/mg}$  protein, respectively,  $p < 0.01$ ) (**Figure 2**).

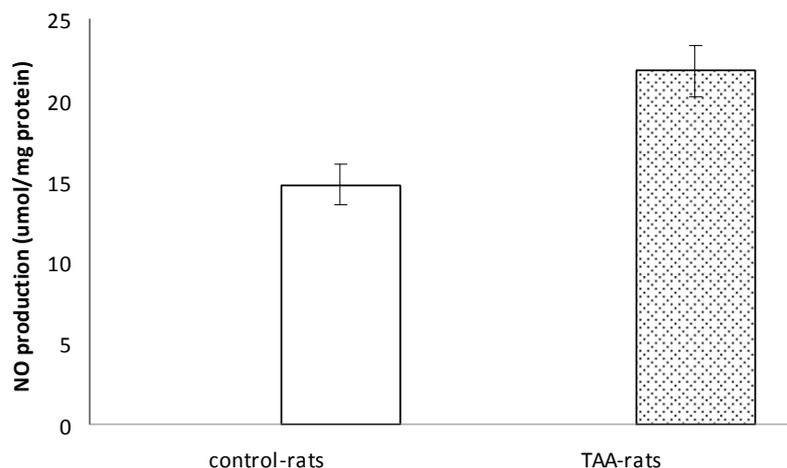
Measurement of low molecular dialdehydes (MDA and HNE) and carbonyl groups, biomarkers of lipid peroxidation and protein oxidation, respectively, were carried out by standardized methods as described in Materials and Methods. Results are shown in **Table 1**. The oxidation of lipids has been analyzed using two different methods: malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE). In both cases, we found higher levels in TAA-rats than in control-rats ( $0.062 \pm 0.006 \mu\text{mol}$  MDA/mg protein versus  $0.021 \pm 0.004 \mu\text{mol}$  MDA/mg protein,  $p < 0.01$ ; and  $0.018 \pm 0.003 \mu\text{mol}$  HNE/mg protein versus  $0.008 \pm 0.002 \mu\text{mol}$  HNE/mg protein,  $p < 0.01$ , respectively).

**Table 1.** Ammonia concentration in plasma and cortex. Lipid peroxidation and protein oxidation in the cortex of control- and TAA-rats.

	Control-rats	TAA-rats	p
<b>Ammonia:</b>			
Plasma ( $\mu\text{mol/l}$ )	$97.50 \pm 9.23$	$303.98 \pm 13.56$	$<0.01$
Cortex ( $\mu\text{mol/g}$ tissue)	$3.44 \pm 0.56$	$8.39 \pm 1.07$	$<0.01$
<b>Lipid peroxidation:</b>			
MDA ( $\mu\text{mol/mg}$ protein)	$0.026 \pm 0.004$	$0.082 \pm 0.010$	$<0.01$
HNE ( $\mu\text{mol/mg}$ protein)	$0.016 \pm 0.002$	$0.039 \pm 0.004$	$<0.01$
<b>Protein oxidation:</b>			
C = O (nmol/mg protein)	$2.72 \pm 0.24$	$5.32 \pm 0.47$	$<0.01$



**Figure 1.** Active oxidants production in control- and TAA-rats.



**Figure 2.** NO production in control and TAA-rats.

Protein oxidation has been studied by quantification of carbonyl groups, by 2,4-dinitrophenylhydrazine (DNPH) method, observing a higher level of protein oxidation in TAA-rats compared to *sham*-rats ( $5.30 \pm 0.57$  nmol/mg protein versus  $2.72 \pm 0.24$  nmol/mg protein,  $p < 0.01$ , respectively).

These results show that hyperammonemia promotes oxidative stress in the brain of TAA-rats, but they do not reveal the mechanisms and proteins involved in the physiopathology of HE.

### 3.2. 2D-DIGE Analysis of Hyperammonemic Rat Brain Cortex Proteins

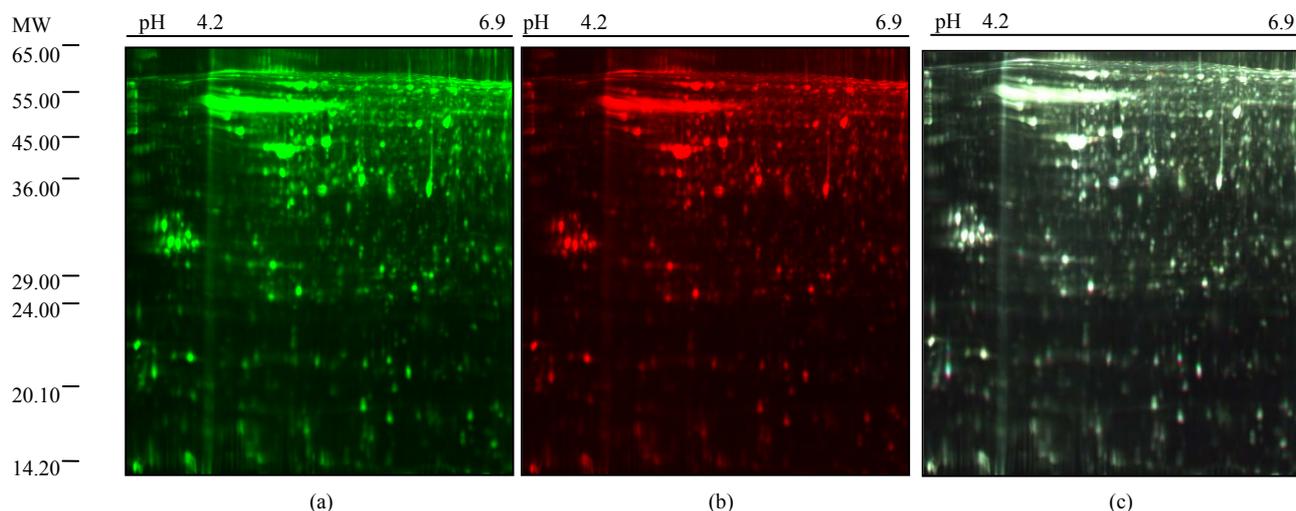
In order to identify proteins involved in the physiopathology of HE, we have studied differential protein expression in brain cortex from TAA- and control-rats. Differential protein expression pattern has been analyzed by 2D-DIGE technique using minimal labeling with three fluorophores (Cy2, Cy3 and Cy5). Contrary to conventional protein staining such as Coomassie brilliant blue (CBB) or silver staining, fluorescent tagging of the entire brain cortex proteins drastically increases the number of detectable protein spots in standard 2-DE. A previous proteomic study of brain rat brain cortex proteins using CBB G-250 resulted in the detection of 636 individual 2-D spots (results not shown). The fluorescent DIGE analysis of brain cortex protein from TAA- and control-rats described here resulted in the visualization of  $2896 \pm 18$  spots (**Figure 3**). Hence, the DIGE-based proteomic profiling study of brain cortex rat proteins is approximately 4.5-fold more sensitive as compared to conventional protein labelling techniques. In addition, gel-to-gel variations in the 2-D protein distribution pattern are greatly reduced due to the simultaneous analysis of two different proteomes and a pooled standard on the same slab gel [21-23]. Thus, around 3000 differentially labelled brain cortex proteins can be analyzed *per* gel

using 2D-DIGE technology, which is far superior to conventional nonfluorescent methods. In order to prevent the introduction of potential artifacts due to subcellular fractionation, this study employed total brain cortex extracts as starting material for our comparative proteomic profiling study. Consequently, the brain cortex proteins analyzed here represents major soluble proteins and therefore does not take into account potential changes in integral receptors and other non-soluble components. **Figure 3** illustrates the representative protein spot pattern obtained for a 2D-DIGE experiment, showing the spots obtained for the control-rats cortex proteins (Cy3 excited, image A) and the TAA-rats cortex proteins (Cy5 excited, image B), respectively. As shown in the superposition of protein spot pattern obtained by labeling with Cy3 and Cy5 (image C). The analysis of these results by the DeCyder™ Software, reveals that no statistical differences between protein expressions in the cortex of TAA- and control-rats were observed.

## 4. DISCUSSION

To verify that after 12 weeks TAA-treated rats ( $n = 8$ ) were hyperammonemic, ammonia concentration in plasma and brain of control- and TAA-rats was assessed; obtaining the following results:  $96.6 \pm 12.3$   $\mu\text{moles/l}$  and  $356.6 \pm 32.3$   $\mu\text{moles/l}$ ,  $p < 0.01$ , in plasma and  $0.36 \pm 0.05$   $\mu\text{moles/g}$  tissue and  $0.97 \pm 0.13$   $\mu\text{moles/g}$  tissue,  $p < 0.01$ , in the brain, respectively. These results show that ammonia levels in plasma and total brain tissue were higher in TAA-rats compared to control-rats, 3.12- and 2.43-fold higher, respectively.

The presence of oxidative stress in the brain of 12 weeks-old TAA-rats was confirmed by measurement of global active oxidants production, in a heterogeneous mixture of neurons, glial and microglial cells from TAA- and control-rat brain. Active oxidants production was measured using DCFDA as a probe. Active oxidants



**Figure 3.** 2-D gel images of rat cortex labeled with minimal CyDye DIGE fluorophores. Control-rats sample labeled with Cy3, (a); TTA treated-rats sample labeled with Cy5, (b); Superposition of images A and B, (c).

production in TAA-rats was 2.7-fold higher than control-rats (**Figure 1**). The measurement of low molecular dialdehydes (MDA and HNE) and carbonyl groups, biomarker of lipid peroxidation and protein oxidation, respectively, were found to be significantly increased in TAA-rats compared with control-rats (**Table 1**). This result reflects the presence and action of oxidative stress in the brain of 12 week-old TAA-rats, where lower enzymatic antioxidant defense has been described too [24, 25].

These results agree with other studies that show that high ammonia concentration exposure induces oxidative stress in rat astrocyte cultures and mouse brain slices *in vitro* [14,26,27], and *in vivo* in the rat brain [24].

Metabolic, energetic and signal transduction-related alterations observed in HE and hyperammonemic rats [4] has been related with altered movements and psycho-intellectual activities. These alterations might be explained in part by differential expression of proteins and enzymes in the cortex of hyperammonemic rats [28-30], a brain area related with psychomotoric disturbances. Therefore, we study protein expression in the cortex of TAA-rats, an animal model of chronic HE, and *sham*-rats by a proteomic approach based on 2D-differential in gel electrophoresis (2D-DIGE).

2D-electrophoresis is an important proteomic tool, where thousands of proteins spots can be visualized, resulting in a global view of the state of a proteome [31], and comparing the 2D spot patterns from different samples (control- and TAA-rats cortex, for example), changes in individual proteins abundance can be detected and quantified; allowing the identification of protein markers that are characteristic for a specific physiological or pathological state of a cell or a tissue [32]. However, a limitation on comparative 2-D analysis is the high degree

of gel-to-gel variation in spot patterns that makes it difficult to distinguish any true biological variation from experimental variation [33]. A method that minimized these drawbacks is differential in gel electrophoresis (DIGE), where samples are labeled prior to electrophoresis with spectrally resolvable dyes Cy<sup>TM</sup>2, Cy3 and Cy5. Samples are then mixed prior to EIF and resolved on the same gel [21]. The fluorophores are structurally similar and undergo nucleophilic substitution with the  $\epsilon$ -amino group of lysine residues forming an amide. The fluorescent dyes have very similar molecular masses and are positively charged to match the charge that is replaced on the lysine residue. This charge/mass matching ensures that all the samples essentially comigrate to the same point during electrophoresis. This ensures that, when the dye:protein ratio is low, protein molecules are only labelled with a single dye molecule [22], refining greatly the detection of changes at the protein level between samples.

Variation in spot intensity due to experimental factors, such as protein loss during sample entry into the strips, will be the same for each sample within a single DIGE gel. Therefore the relative amounts of a protein between samples in a gel will be unchanged. With conventional "one sample per gel" 2D techniques, samples to be compared are separated independently in different gels, and consequently, spot migration and intensity will differ for each gel and sample in an experiment adding to the overall experimental variation.

The analysis of the spots (**Figure 3**) showed no statistically significant differences in expression among the 2896 resolved spots by DIGE method in the cortex of TAA-treated rats and control-rats. Although these results clearly indicate that no statistically significant differences ( $p < 0.05$ ) were observed between TAA-treated

rats and *sham*-rats, it should be stated that systematic differences for 14 spots were observed by DIGE study. Although these slightly differences could not be considered as the main causes of HE, its role should also not be excluded.

These results support the idea that oxidative post-translational modifications are implicated in HE physiopathology, and have leaded us to consider the hypothesis that the cause of HE lies in post-translational modifications rather than in differential expression of proteins. We are currently working on this hypothesis.

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