ABSTRACT: Vitamin B2 deficiency associated with normal dietary intake has been reported in patients with Parkinson's disease (PD), suggesting impaired absorption of this micronutrient. Elevated red meat consumption was thought to contribute as a triggering factor, as the catabolism of hemin (a neurotoxic substance) requires vitamin B2 (Coimbra & Junqueira, 2003). This study tested this hypothesis by verifying the effects of dietary riboflavin restriction associated with hemin administration on rat brain. After 8 months of riboflavin restriction, riboflavin deficiency with or without oral administration of hemin (assessed by erythrocyte glutathione reductase activity) did not impair motor function or spatial learning; neither altered the volume of substantia nigra or brain concentrations of total glutathione. Partial dietary restriction of riboflavin may failed to induce oxidative stress in the rat brain and dopaminergic degeneration in the rat substantia nigra as suggested to occur in humans by Coimbra & Junqueira, (2003), possibly due to an intact mechanism of nutritional privilege that preserves riboflavin content in the normal rat brain during deficiency states. Contrasting, polymorphic enzymes or receptors involved in the human cellular uptake of riboflavin may conceivably impair the transport of this micronutrient not only through the intestinal wall and renal tubules, but also in the brain of PD patients, thereby annulling the nutritional privilege of the nervous system.

KEY WORDS: FAD, Glutathione, Hemin, Parkinson's disease, Riboflavin, Substantia nigra.

Abbreviations: CNS (central nervous system), CSF (cerebrospinal fluid), DA (dopamine), EGR-AC (erythrocyte glutathione reductase activation coefficient), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), PD (Parkinson's disease), Rbf (riboflavin) SN (substantia nigra), SNpc (SN pars compacta), GSH (total glutathione), GSSG (oxidized glutathione).

INTRODUCTION

The impact of long lasting riboflavin (Rbf) deficiency on the adult CNS is not fully understood, since selective Rbf deficiency is not or hardly ever seen in human diets, and experimental studies have focused mainly on the developing brain. Considering that Rbf is a major building block for flavin coenzymes (FMN and FAD), which act as co-factors or prosthetic groups for fundamental flavo-enzymes such as cytochrome P450 reductase, glutathione reductase, and mitochondrial complexes I and II (among others), low Rbf status may negatively impact the adult brain in the long run (Hoppel and Tandler, 1990; Hara and Taniguchi, 1982; Hustad et al, 2002; Powers, 2003). In addition, there is reasonably good evidence that poor Rbf status interferes with iron handling (Powers, 2003), and altered iron metabolism is a major feature in PD (Gottz et al., 2004).

Laboratory indicators of low vitamin B2 status, in spite of a normal dietary content of Rbf have been reported in PD patients (Coimbra and Junqueira, 2003), suggesting an impaired absorption of this micronutrient. Those authors reported elevated dietary red meat by PD patients and proposed that a large consumption of these products could contribute as a triggering factor for neurodegeneration of SN dopaminergic cells, as the catabolism of
hemin - a lipid-soluble neurotoxic component of red meat (Huang et al., 2002; Letarte et al., 1993; Kumar and Bandyopadhyay, 2005; Everse and Coates, 2005), requires vitamin B2 (Coimbra and Junqueira, 2003). Those authors also reported that normalization of vitamin B2 status by administering high doses (30 mg) of Rbf 3 times per day and elimination of dietary red meat normalized laboratory results and were associated with progressive and sustained motor improvement throughout the first 6 months of observation.

On the other hand, obtaining appropriate animal models is crucial for unveiling the pathophysiology of PD and for the development of disease-modifying therapies. According to Emborg (2004), “an ideal animal model of disease can be described by presenting behavioral signs and pathology that resemble the disease, including its time course. The closer the similarity of a model is to PD, the higher the predictive validity for clinical efficacy. Modeling PD is limited by our knowledge of what causes the disease”.

Based on those clinical findings, this study aimed at developing an animal model of PD by verifying the effects of dietary Rbf restriction associated with high oral doses of hemin on rat SN dopaminergic cells, motor activity and redox state of brain glutathione, contributing to the discussion on the problematic of animal modeling of PD based on clinical findings, pursuing the optimal interaction between basic and clinical research.

MATERIAL AND METHODS

Animals

The animal experiments were approved by the ethics committee of the Federal University of São Paulo (UNIFESP), following the “Principles of laboratory animal care” (NIH publication no. 86–23, revised 1985). Adult (3-month old) male Wistar rats of the Charles-River strain were maintained on a 12hr light/12hr dark cycle (7:00a.m. /7:00p.m.) and given free access to food and water. Fifty-six rats were allocated into 4 groups given special research rodent diets purchased from Harlan Teklad (USA): Group A: purified (AIN) diet; Group B: Rbf deficient diet (1μg/g); Group C: purified (AIN) diet plus bovine hemin chloride 3.5mg.kg−1.day−1; Group D: Rbf deficient diet plus bovine hemin chloride 3.5mg.kg−1.day−1. The degree of dietary Rbf deficiency was chosen as to avoid mortality during prolonged study periods (Prentice and Bates, 1981). Coprophagy was minimized by fitting rat cages with metal grids that allow fecal pellets to pass through to the floor of the cage.

Assessment of Rbf and iron status

For total serum iron, animals were anesthetized with halothane and mechanically ventilated, when 2ml of blood were collected from the left ventricle into a dried tube immediately before anticoagulation with heparin for perfusion-fixation, and centrifuged at 2000rpm for 10 minutes. Total serum iron was analyzed according to Artiss et al. (1981) and Siedel (1984) through spectrophotometry (ADVIA 1650, Japan) using a kit for iron dosage (Bayer, USA). Iron was dissociated from transferrin in an acid medium and reduced to ferrous iron, which is complexed with a chromogen (ferrozin) to produce a chromophore with maximal absorbance at 571 nm. Rbf status was assessed by determination of EGRAC (Sauberlich et al., 1972) in red blood cell lysates (Beutler, 1975). Additional 2 ml of heparinized blood was collected from the heart prior to perfusion-fixation for this purpose.

Analysis of volume of Substantia Nigra pars compacta (SNpc)

After 8 months of survival the animals were anesthetized with halothane, mechanically ventilated, heparinized and perfusion-fixed with 0.9% NaCl followed by phosphate-buffered 4% formaldehyde. Brains were allowed to fix in situ for 16–24 h, and then removed, embedded in agar and cut with a vibratome ("VT 1000 S", Leica, Germany), into 40-μm-thick coronal sections throughout the entire SN. Every 10th section was sampled systematically with a random start between sections 1 and 5, mounted into gelatinized onto slides and stained with celestine blue.

The image of SN was captured with a digital camera (Spot RT Slider) under the objective of 4x (Nikon E600) and was then transferred to a CRT 19" monitor via an image analysis software (Image-Pro Plus, Media Cybernetics), where the boundaries of SNpc were delimitated. An acetate containing points in a grid pattern was then overlaid on the image for the employment of Cavalieri’s technique (Gundersen et al., 1988) for counting the points located inside the boundaries of SNpc. The total volume of SNpc, \( V_{SNpc} \), was estimated using point-counting and the Cavalieri principle (Gundersen and Jensen, 1987): \( V_{SNpc} = k × t × a(p) × \sum P \), where “\( k \)” = inverse fraction of sampled sections, “\( a(p) \)” = the area per point on the test grid corrected for magnification, and “\( \sum P \)” = total number of test points hitting all SNpc profiles.

Immunohistochemical analysis

Vibratome sections representative of rostral, medium and caudal portions of SN not used for volume estimation of SNpc were immunostained for synuclein (Santa Cruz, 1:1000), ubiquitin (Dako, 1:1000), transferrin receptor (Santa Cruz, 1:800), transferrin receptor (Santa Cruz, 1:800), and tyrosine hydroxylase (Sigma, 1:1500) as described elsewhere (Sinigaglia-Coimbra et al., 2002). Visualization was achieved with the streptavidin-biotin method (rat LSAB, DAKO), using liquid 3,3ʹ-diaminobenzidine tetrahydrochloride (DAB, DAKO) as the chromogen.

Glutathione Measurements

Both forms of glutathione (total:GSH and oxidized:GSSG) were measured in total brain tissue (Tietze, 1969). After perfusion with 0.9% NaCl solution at 4°C under halothane anesthesia and mechanical ventilation, the brains were removed from skull and homogenized in 0.5 M perchloric acid (1:50, v:v). The homogenate was centrifuged at 5,000 rpm for 20 min, at a temperature of 4–5 °C. An aliquot of the supernatant was neutralized with 1.75 M K3PO4 and centrifuged at 12,000 rpm for 3 min, and the supernatant was used for both GSSG...
and GSH determinations. For GSH assay, an aliquot of the supernatant was added to the standard glutathione assay mixture containing 0.1 M phosphate/0.001M EDTA buffer (pH=7.0), NADPH (4mg/ml), DTNB (1.5mg/ml) and glutathione reductase (6U/ml). The GSH assay’s reaction was measured spectrophotometrically at 412 nm for 3 minutes (25°C). For GSSG determination, another aliquot of the supernatant was added to a standard mixture containing 0.5 M phosphate buffer (pH=6.8), NADPH (10mg/ml) and glutathione reductase (20U/ml). The GSSG assay’s reaction was measured spectrophotometrically at 340 nm for 16 minutes (30°C). Values were expressed in micromols per gram of tissue.

**Motor/Spatial learning evaluation**

All groups were tested for reference memory with the Morris water maze task (Morris et al., 1982). Testing occurred in a 2.0 m diameter black pool. An overhead camera was connected to a video monitor and a computer running the software Ethovision 2.3 (Noldus Information Technology, Netherlands) used to track the rat swimming path, and to calculate the path length and the time (latency) spent to reach an invisible (black) submersed platform. The water was leveled at 1.0 cm above the hidden platform and maintained at 26.0±1.0 °C.

Each animal was tested four times a day for eight consecutive days. For scoring purposes, the pool was broken up into 4 equal sections, arbitrarily identified as northeast (NE), southeast (SE), northwest (NW), and southwest (SW) quadrants. The platform was placed in the middle of the NE quadrant, for all testing. The animals were released into the pool from each of 4 starting locations daily, in a pattern that was randomly determined prior to testing. The starting points corresponded to north, east, west, and south, based on the position of the arbitrary quadrants.

For every trial, the animal was placed in the pool facing the wall. Animals were allowed 120 seconds to find the platform. If they were unable to find the platform in that time, they were guided to it by hand. They were allowed to remain upon the platform for 30 seconds, and were then removed. Visual cues were available within the testing room. A minimum of 5 min elapsed between trials, when the animal was placed under a heating lamp, on an elevated platform in the testing room. All tests started by 9:00 AM, and the order in which the animals were tested was randomly changed.

**Data analysis**

Repeated measures analysis of variance was used for analysis of latency, path length and velocity, followed by Dunn’s post-hoc test. Mean latency ± SEM of each day (session) was obtained for the purpose of data representation. Data related to total and oxidized glutathione (GSH and GSSG, respectively), total serum iron and erythrocyte glutathione reductase activation coefficient (EGRAC) were analyzed with the one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. Significance testing for SNpc volumes was performed using the Kruskal-Wallis test, followed by Mann-Whitney’s U test.

### TABLE 1. Riboflavin status (EGRAC measured in erythrocytes) and total serum iron observed in rats fed a normal purified or Rbf deficient diet for 8 months, with or without hemin treatment (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>PURIFIED</th>
<th>PURIFIED + HEMIN</th>
<th>RBF DEFICIENT</th>
<th>RBF DEFICIENT + HEMIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGRAC (ref val 1.00 - 1.20)</td>
<td>1.12 ± 0.039</td>
<td>1.20 ± 0.083</td>
<td>1.44 ± 0.022</td>
<td>1.632 ± 0.052</td>
</tr>
<tr>
<td>Total serum Iron (ref val 49-160)</td>
<td>155 ± 19.78</td>
<td>189 ± 45.96</td>
<td>184 ± 46.63</td>
<td>181.8 ± 35.45</td>
</tr>
</tbody>
</table>

### TABLE 2. Total (GSH) and oxidized (GSSG) forms of glutathione brain levels, as well the ratio GSH/GSSG observed in rats fed a normal purified or Rbf deficient diet for 8 months, with or without hemin treatment (mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>PURIFIED</th>
<th>PURIFIED + HEMIN</th>
<th>RBF DEFICIENT</th>
<th>RBF DEFICIENT + HEMIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH whole brain (mcg/mol)</td>
<td>1.03 ± 0.35</td>
<td>0.99 ± 0.40</td>
<td>0.95 ± 0.20</td>
<td>1.09 ± 0.30</td>
</tr>
<tr>
<td>GSSG whole brain (mcg/mol)</td>
<td>2.40 ± 2.05</td>
<td>2.50 ± 1.86</td>
<td>1.84 ± 1.88</td>
<td>2.31 ± 2.14</td>
</tr>
<tr>
<td>GSH striatum (mcg/mol)</td>
<td>1.10 ± 0.33</td>
<td>0.99 ± 0.40</td>
<td>0.97 ± 0.25</td>
<td>1.09 ± 0.38</td>
</tr>
<tr>
<td>GSSG striatum (mcg/mol)</td>
<td>0.60 ± 0.50</td>
<td>0.45 ± 0.47</td>
<td>0.24 ± 0.25</td>
<td>0.25 ± 0.26</td>
</tr>
<tr>
<td>GSH/GSSG whole brain (mcg/mol)</td>
<td>2.40 ± 2.13</td>
<td>3.65 ± 2.26</td>
<td>1.78 ± 1.75</td>
<td>2.53 ± 2.58</td>
</tr>
<tr>
<td>GSH/GSSG striatum (mcg/mol)</td>
<td>0.51±0.43</td>
<td>0.83±0.65</td>
<td>0.27±0.36</td>
<td>0.12±0.13</td>
</tr>
</tbody>
</table>

### RESULTS

Only the animals fed a Rbf-deficient diet plus hemin supplementation showed a significantly altered Rbf status, although both Rbf-deficiency and Rbf- deficiency plus hemin groups showed EGR-AC levels above the upper limit of normal range of 1.20 (Hustad et al., 2002) (Figure 1 and Table 1). This parameter basically demonstrates the systemic status of Rbf, and may not accurately reflect the Rbf status in the brain tissue. No statistical difference for total serum iron was observed (figure 2). Accordingly, brain glutathione analysis (GSH, reduced; GSSG, oxidized and GSSG/GSH ratio) did not differ
among groups (figure 3 and Table 2). Volume estimation of SNpc showed no statistical difference among groups (figure 4). Repeated measures analysis of variance of motor and spatial learning data from the experimental groups revealed no statistical differences (figure 5). All groups improved their performance along the periodic testing during the 8-month survival, indicating that animals presented no signs of impairment in motor or spatial learning.

Finally, neither dietary restriction of Rbf nor hemin chloride administration, alone or in combination, could affect pattern of immunostaining (for any of the listed proteins) observed in the brains of rats fed a purified normal diet (data not shown).

FIGURE 1. Rbf status (as assessed by EGR-AC) in animals fed 2 different (purified or Rbf-deficient) diets, with or without hemin treatment, for 8 months. The dashed line indicates the maximum EGR-AC level considered as normal. Groups receiving Rbf-deficient diet presented EGR-AC above 1.20, indicating low Rbf status. Although both groups receiving Rbf deficient diet showed augmented EGR-AC, only group Rbf deficient + hemin was statistically different from the animals fed a purified diet (ANOVA, followed by Tukey post hoc test). Rbf = riboflavin; EGR-AC = erythrocyte glutathione reductase activation coefficient.

FIGURE 2. Total serum iron in animals fed 2 different (purified or Rbf-deficient) diets, with or without hemin treatment for 8 months. No significant differences were observed (ANOVA). Rbf = riboflavin.

FIGURE 3. GSH (A) and GSSG (B) levels in the whole brain tissue and striatum of animals fed 2 different (purified or Rbf-deficient) diets with or without hemin treatment for 8 months. In (C), the GSH/GSSG ratio in whole brain and striatum homogenates of groups receiving purified or Rbf-deficient with or without hemin at 8-month survival. No significant differences were observed (ANOVA). GSH = reduced glutathione; GSSG = oxidized glutathione; Rbf = riboflavin.
FIGURE 4. Volume estimation of substantia nigra pars compacta in animals fed purified or Rbf-deficient diets, with or without hemin treatment, for 8 months. No significant differences were observed (Kruskal Wallis test). Rbf = riboflavin.

FIGURE 5. Motor and spatial learning performance during the reference memory test at the Morris’s water maze of animals fed purified or Rbf-deficient diets, with or without hemin treatment for 8 months (series of 4 daily trials during 8 consecutive days). A (mean escape latency) and B (mean path length) demonstrate spatial learning capacity, while C (mean velocity) demonstrates motor capacity. No significant differences were observed (ANOVA for repeated measures). Rbf = riboflavin.

DISCUSSION
Our study showed that Rbf dietary restriction associated to oral administration of hemin chloride (in contrast to Rbf dietary restriction alone), caused systemic Rbf deficiency but did not induce chronic neuronal demise or the appearance of Lewy bodies in the adult rat SNpc. That treatment paradigm neither altered serum iron levels or brain GSH and GSSG concentrations. Accordingly, no behavioral or motor abnormalities were detected during the development of spatial navigation memory.

In contrast, Coimbra and Junqueira (2003) observed improved motor function during the initial 3 months of high-dose oral Rbf replacement (plus elimination of dietary red meat) followed by a trend to clinical stabilization. The therapeutic effect of xenobiotics requires the support of randomized clinical trials to be widely accepted. In contrast, the results of the open-label study by Coimbra and Junqueira (2003) are strongly supported a low basal Rbf status consistently found in their PD patients. In addition, correction of Rbf status paralleled the clinical response, and the latter did vanish within 6 months of observation despite sustained treatment – as it would be expected from placebo effects in PD patients, according to the study by Goetz et al (2000), who observed placebo-associated motor improvements in only 16% of 105 PD subjects evaluated at 4, 12 and 24 weeks of treatment, and demonstrated that none of them showed improvement at all three evaluations. Furthermore, the identification of a consistent and correctable metabolic change capable of driving the degeneration of dopaminergic neurons in PD patients creates an undeniable ethical impediment against doing clinical trials using untreated humans as controls. This issue further stresses the need for well-conducted animal experiments that could definitely support or rule out the pathophysiological importance of Rbf deficiency for sporadic PD.

In addition, the interest in pursuing experimental studies on this issue is further strengthened by the extensive overlap between the pathophysiological features of PD and the metabolic consequences of Rbf deficiency. Not only iron metabolism is disrupted in both situations (as introductorily stated here), but other classical characteristics of PD may be enabled or fully explained by poor Rbf status, including impaired activity of mitochondrial enzyme complexes I and II (Bindoff et al., 1989; Mizuno et al., 1989; Parker et al., 1989; Schapira et al., 1990; Bindoff et al., 1991; Cardellach et al., 1993; Blin et al., 1994), GSH depletion (Riederer et al., 1989; Dexter et al., 1994), and selective degeneration of dopaminergic neurons. Complexes I and II are respectively FMN- and FAD-dependent (Nelson and Cox, 2000), and GSH is regenerated from GSSG in a reaction catalyzed by a flavo-enzyme – GSH reductase (Hustad et al., 2002). Hemin is produced during digestion of hemeproteins and readily absorbed, reaching the brain tissue if not properly degraded through a Rbf-dependent process (Hara and Taniguchi, 1982; Hustad et al., 2002; powers, 2003; Gotz et al., 2004; Coimbra and Junqueira, 2003). The excess of cytosolic free iron observed in the SN neurons of PD patients (Logroscino et al., 1998) may also arise from the fact that iron deposition into
ferritin is also a Rbf-dependent mechanism (Adelekan and Thurnham, 1986). As a consequence of Rbf deficiency, excess of superoxide anion production by mitochondria and GSH depletion combine to accumulate hydrogen peroxide, thereby enhancing Fenton reaction in the presence of free iron. The resultant hydroxyl radical reacts with dopamine to yield 6-hydroxy-DA – a potent neurotoxin found in the urine and CSF of PD patients (Andrew et al., 1993), ultimately leading to selective demise of dopaminergic neurons.

An ideal animal model of PD should resemble the behavioral signs and pathology of the disease. The closer the similarity of a model is to PD the higher the predictive validity of experimental results for clinical trials (Emborg, 2004). However, current efforts to develop of animal models of PD are limited by incomplete knowledge of what causes the disease (Emborg, 2004). Therefore, the participation of Rbf in most biochemical pathways known to be disturbed in PD should be properly valorized, in attention to the principle of parsimony.

Failure to induce brain GSH depletion or chronic demise of dopaminergic neurons in SNpc by dietary restriction of Rbf with or without hemin treatment (in spite of achieving a systemic Rbf deficiency) indicates that local Rbf status remained relatively unaffected, possibly due to the activity of powerful homeostatic mechanisms in the CNS of genetically normal rats (Spector, 1980). In contrast, an inherited impairment of the cellular uptake mechanism may limit the transport of Rbf not only through the intestinal wall and renal tubules, but also through the blood-brain barrier in PD patients. The potential role of Rbf deficiency documented in PD patients deserves more thorough experimental investigation, and the use of a Rbf antagonist capable of crossing the blood-brain barrier in future experimental studies on the effects of Rbf deficiency may be required, either associated with dietary iron overload or other neurotoxic agents (Collins et al., 2002) potentially capable of triggering PD in genetically predisposed individuals.

ACKNOWLEDGEMENTS

This work was supported by FAPESP (Grant 04/05388-8). Janise Dal Pai and Andrea A Borges are CAPES and Fapesp fellows, respectively.

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