

Riboflavin ameliorates the L-NAME induced brain injury

A Riboflavina na melhora da lesão cerebral induzida por L-NAME

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Abstract— Introduction: Oxidative stress has been associated with the pathogenesis of vascular disorders as hypertension and stroke. Studies have been carried out to identify preventive alternative therapies, including vitamins with antioxidant power. Methods: Twenty-four adult male rats (SHR-sp) were subdivided into four groups with six rats (n=6) each: control (not treated), riboflavin (B2 treated), B2 plus L-NAME, and L-NAME. Individual neurologic outcome status was appraised through maze, balance and motor tests. Oxidative stress serum markers (Homocysteine and Malonic Dialdehyde) were determined for each group. The hypotensive effect ($p < 0.05$) observed with L-NAME: $196.5 \pm 1.97 \text{ mmHg}$ (L-NAME plus B2), $195 \pm 3.3 \text{ mmHg}$ (B2); $235.3 \pm 2.16 \text{ mmHg}$ (control), $233.7 \pm 4.50 \text{ mmHg}$ (L-NAME). There was neuroprotective effect of riboflavin in the response to those neurological tests. Regarding oxidative stress markers, animals treated with riboflavin showed lower values ($p < 0.05$), suggesting better protection. Conclusions: Even under NO synthase inhibition, riboflavin enhanced neurological outcome status and reduced systolic pressure levels. Discoveries suggest riboflavin might become a preventive and regenerative strategy of stroke management.

Keywords— Oxidative Stress, riboflavin, L-NAME, neurological tests.

I. INTRODUCTION

Cerebrovascular accidents (CVA) have a peak incidence between 6th and 7th decades of life when added to cardiovascular and metabolic alterations related to age^{10,18}. However, a stroke may occur earlier and be related to other risk factors such as diabetes, hypertension, obesity, immune and inflammatory diseases²⁰. Recent global studies revealed an increased incidence of strokes worldwide, surrounding 10 million cases in incidence⁵.

Regarding this, there is a strong evidence implicating oxidative stress in the pathogenesis of vascular disorders in general. The reaction of free radicals with macromolecules starts an oxidation process triggering an inflammatory reaction which leads to both endothelial injury and decreases the elasticity of large and small vessels causing hypertension and stroke¹⁵. Thus, a number of studies have been carried out to identify alternative therapies which include the possible action of vascular endothelial protective nutrients, including vitamins with antioxidant properties^{3,16}. In addition, some authors have reported a physiological downregulation of riboflavin kinase in SHRSP rats²³, a known experimental model for the study of severe hypertension and stroke as well.

Those findings putting together bring up the riboflavin (vitamin B2) due to its role on the mechanism of oxidation-reduction and the metabolism of homocysteine⁶. Besides that, the possibility that riboflavin might be involved in nitric oxide synthesis increases the interest in the investigation of riboflavin's effects in brain injury models involving oxidative stressors such as L-Nitroarginine Methyl Ester (L-NAME).

In fact, nitric oxide has been recognized as both: an endothelium-derived relaxing factor (EDRF) substance and neurotransmitter¹⁷. The inhibition of nitric oxide synthase (NOS) has been related to brain injury as well¹¹. Therefore the aim of this study was to investigate the possible effects of riboflavin (B2) chronic supplementation on neurologic performance of spontaneously hypertensive stroke-prone rats (SHR-sp) previously submitted to L-NAME.

II. MATERIALS AND METHODS

Animals and supplementation

Twenty-four adults (18 weeks) male spontaneously hypertensive stroke-prone rats (SHR-sp), obtained from the colony of the bioterium of the Federal University of the State

of Rio de Janeiro, were kept in individual metabolic cages in controlled conditions: temperature ($21 \pm 2^\circ \text{C}$), humidity ($60 \pm 10\%$), air exhaustion cycle (15 min / h) and 12 h-dark/light cycle (artificial lights, 7 am-7 pm) and fed a standard diet Nuvilab (Nuvital®) plus water *ad libitum*.

After a baseline period of 10 days, the rats were randomly subdivided into four groups of six animals each: Group 1 - Control (receiving vehicle: water), Group 2 - Riboflavin(B2) Treated (10mg/Kg of body weight) (Sigma®-R4500), Group 3- Riboflavin(B2) plus L-Name (50mg/kg) (N5751, Sigma®) and Group 4- L-Name isolated. The duration of Treatment was 28 days and riboflavin was administered by oral gavage and L-NAME in drinking water. All the procedures were carried out in accordance with the conventional guidelines for experimentation with animals (NIH Publication No. 85-23, revised 1996). The experimental protocols used in this study were approved by the Ethics Committee for Use Animal Experimentation (CEUA) at the Federal University of Rio de Janeiro State, Protocol N.2016-2.

Physiological parameters

The animals maintained in metabolic cages were submitted to the daily evaluation of water and food intake, body weight, diuresis and physical aspects: distribution and coloring of hair, bleeding, stains, cracks, opacification, and coloring of mucous. The behavioral aspects and motor-sensory parameters investigated were also following Kolb & Whishaw methodology²². Systolic blood pressure was determined through the non-invasive method of plethysmography⁷.

Neurological tests

The sensory-motor skills of the animals were assessed daily from the basal period:

- Memory test and spatial orientation (Maze test). The animals were initially trained for a week in a maze for the development of memory and spatial orientation. After this step, the treated rats and control groups received their treatment for 4 weeks and the rats were retested. The animals were placed in a one-way point of a maze with dimensions 30 X 55 X 55 cm and should find the exit. When the animal ran all the way, the task runtime was recorded. If the animal took more than 120 sec to travel the path, the test was stopped and the trademark of 120 sec was recorded.

- Balance test- Inclined plane: the balance bar is a wooden structure 5mm larger, whose function was to assess the time at each animal could support its own weight through the power of the forelimb placing holding it at the bar. The time the rats took the bar holding was timed.
- Motor Tasks: This test was performed by measuring the sensitivity of the animal to pain in response to application of heat (water at 70 ° C) at the tail end of the animal where the response time to the stimulus is timed using a sport timer of the brand “Miky” (adaptation of the method SDI Tail Flick Analgesia Meter, the San Diego Instruments - USA)
- Behavioral alterations and TIA and stroke signals: The animals were handled daily and any evidence of neurological sensory, motor or behavior alterations was recorded.

Blood Collection

At the end of treatment, the animals under anesthesia with sodium pentobarbital (60 mg/Kg of body weight) had the blood collected by puncture of the caudal vein, for biochemical analysis.

For the sacrifice, the same drug was used, at high dose (100mg/Kg of body weight) until the absence of vital signs and then the liver was removed for macroscopic evaluation following Sherle method ¹⁹.

Blood homocysteine (Hcy)

The material used for the determination of homocysteine was frozen plasma obtained from centrifugation of the blood contained in a disposable tube with EDTA anticoagulant. The blood was centrifuged in a centrifuge model CELM Kombat (measurement and calibration Control-Lab), the 3500rpm for 15 minutes. The plasma separated from cells by centrifugation was removed from the primary tube by pipetting with disposable tips and placed in a secondary tube, identified and sterile. The method was used to measure HPLC (High-Performance Liquid Chromatography) using the Shimadzu C18 column with Novapac, reading fluorescence at wavelengths 385 and 515nm.

Malonic Dialdehyde (MDA)

The material used for the determination of malonic dialdehyde serum levels was obtained from the centrifugation of blood collected in a disposable tube without anticoagulant. The blood was centrifuged in a centrifuge

model CELM Kombat (measurement and calibration Control-Lab), the 3500rpm for 15 minutes. The serum separated from cellular components by centrifugation was removed from the primary tube by pipetting with disposable tips and placed in a secondary tube, identified and sterile.

Colorimetric method was used for MDA, using equipment Micronal B442 and thiobarbituric acid as the reagent.

Statistical analysis

Two-way ANOVA model was used to compare the variables among the groups, with a Confidence Interval of 95% threshold being considered statistically significant.

III. RESULTS

Supplementation of B₂ did not alter the biological parameters and general health status of animals. The physical examination together with the macroscopic evaluation of the liver (Table 1) confirmed the absence of toxicity of riboflavin under supraphysiological doses.

In addition, there was a significant role of riboflavin in systolic blood pressure reduction ⁶. Besides that, the significantly ($p < 0.05$) hypotensive effect was observed even in presence of L-NAME: 196.5 ± 1.97 mmHg (L-NAME plus B₂), 195 ± 3.3 mmHg (B₂); 235.3 ± 2.16 mmHg (control), 233.7 ± 4.50 mmHg (L-NAME); (Table1).

Our results indicated a neuroprotective effect of riboflavin. By the end of the experiment (4 weeks), vitamin supplementation has reversed blocking L-Name effect. There was a significant reduction of approximately 22 seconds in memory test, 13 seconds in the balance test and 4 seconds in the motor task.

Neurological observations demonstrated the presence of an ataxia and hemiparesis in one rat from the B₂ plus L-NAME group, but this animal recovered quickly, revealing a picture of a transient ischemic attack. With respect to the L-Name group, all animals were apathetic, irritable to the touch, with bristling hair and two rats showed irreversible ataxia and hemiparesis. In the Control group, the animals were apathetic and irritable to the touch. None of the B₂ group subjects presented visible neurological impairments.

Regarding oxidative stress markers, the animals treated with riboflavin showed significantly ($p < 0.05$) lower values, suggesting a better protection (Table 2).

Physiological general parameters and liver weight injury induced:
of SHR-sp rats Riboflavin treated, Control and L-NAME-

Table 1. Values represent the mean \pm SD of physiological parameters of 6 animals per group.

Physiological Parameters Groups		Body Weight (g)	Diuresis (mL)	Food Intake (g)	Water Intake (mL)	Liver Weight (g/100g bw)	Systolic Blood Pressure (mmHg)
Rats	Control	4.67 \pm 2.23	16.94 \pm 2.14	16.94 \pm 2.14	26.62 \pm 3.63	3.48 \pm 0.21	235.3 \pm 2.16
	B ₂	3.28 \pm 1.27	21.70 \pm 3.53	21.70 \pm 3.53	30.51 \pm 2.12	3.72 \pm 0.24	195 \pm 3.30
	L- NAME	6.68 \pm 5.41	19.09 \pm 3.04	19.09 \pm 3.04	29.97 \pm 6.45	3.31 \pm 0.38	233.7 \pm 4.50
	L-NAME + B ₂	5.43 \pm 3.22	15.34 \pm 4.12	15.34 \pm 4.12	24.86 \pm 4.68	3.75 \pm 0.57	196.5 \pm 1.97

Table 2: Profile of biomarkers associated to an oxidative stress

Markers Group	Hcy (mmol/l)	MDA (nmol/mg)
Control	16.21 \pm 0.63	5.87 \pm 0.38
B ₂	12.45 \pm 0.35 *	4.05 \pm 0.68 *
L-NAME	19.46 \pm 0.56	7.78 \pm 0.87
L- NAME + B ₂	14.67 \pm 0.22 *	5.34 \pm 0.73 *

Table 2. Values represent mean \pm SD of homocysteine and malonic dialdehyde levels of six animals per group. * P < 0.05 was considered significant.

Table 3: Response to Neurological tests

Tests Groups	Memory	Balance test	Motor Task
Control	38''81 \pm 09''36	23''97 \pm 04''27	3'26''31 \pm 0'00''05
B ₂	16''25 \pm 04''51*	19''84 \pm 01''48 *	1'18''29 \pm 0'00''08 *
L-Name	40''41 \pm 04''12	33''77 \pm 02''11	5'33''42 \pm 0'00''05
L-Name + B ₂	18''25 \pm 06''47*	20''44 \pm 02''41*	1'37''19 \pm 0'00''04 *

Table 3. Values represent mean \pm SD of neurological test screening performed in six animals per group. * P < 0.05 was considered significant.

IV. DISCUSSION

Nitric oxide is an important bioactive molecule associated with the physiology of cardiovascular, nervous and immune system. Many studies have shown that nitric

oxide is thought to be involved in synaptic plasticity contributing to learning and memory in several brain areas including the hippocampus.

It is well established that NO synthases metabolize L-arginine to L-citrulline and NO via two consecutive single oxidation reactions. The inhibition of NO synthases by L-NAME has been associated with disturbances in the acquisition, storage, and retrieval of information ². In the presence of L-NAME, different areas of the brain related to memory seem to be injured ¹. The present study demonstrated that the rats from L-NAME group showed a number of neurological disorders such as ataxia, irritability to touch, hemiparesis and loss of memory, in agreement with previous authors ⁹. On the other hand, riboflavin supplementation was able to reverse the dangerous effect of L-NAME both in improving the performance of memory and also in balance ¹⁴.

In addition, the riboflavin supplementation clearly decreased in number and severity the signs of transitory ischemic attacks usually observed in SHR-sp rats. In fact, the very recent discovery of riboflavin brain receptors reinforces the idea that this vitamin may have an important role on brain functioning ²³.

Additionally, riboflavin benefit effects could be in part attributed to the riboflavin antioxidant properties. Such a mechanism of action has been linked to its chemical structure: the presence of a ribitol ring ⁴. The reduction of this ring (FAD, FMN oxidized form) produces the reduced forms of flavoproteins (FMNH₂ and FADH₂) ⁸ which allows the vitamin to have high affinity to react with various substrates, particularly molecular oxygen ^{12,13}.

Another important point to be approached is the fact that riboflavin, as mentioned above, also participates of homocysteine metabolism throughout regulation of metabolic pathways catalyzed by the enzymes methylenetetrahydrofolate reductase (MTHFR) and methionine synthetase ¹⁶. Such an effect was traduced by a sharp reduction of homocysteine blood levels here presented. This effect is extremely important because homocysteine by itself, is considered as a free radical and seems to be implicated in the development of stroke and neuronal damage as well ²¹.

V. CONCLUSION

The present study demonstrates riboflavin 's role in cellular oxidation-reduction, homocysteine metabolism, and antioxidant body defense. Yet, our results showed its action in enhancing the NO synthase action. These properties suggest that riboflavin can be a promising alternative

strategy for the prevention of central nervous system's injuries sustained strokes and transient ischemic attacks.

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