

Article

# Undernutrition Modifies the Antihypertensive and Metabolic Responses of Rostafuroxin in Male Rats: Outcomes on Bodily Na<sup>+</sup> Handling and Renal Na<sup>+</sup>-transporting ATPases

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**Abstract:** The aim of the present study was to investigate whether chronic undernutrition modifies the response to rostafuroxin in juvenile rats. Chronic undernutrition was induced in male rats using a multideficient diet known as Regional Basic Diet (RBD), mimicking alimentary habits in impoverished regions worldwide. Animals were given RBD – or a control/CTRL normal diet for rodents – from weaning to 90 days, and rostafuroxin (1 mg/kg body mass) or vehicle were orally administered during the last 30 days. Undernourished rats became hypertensive at 55–60 days of age (tail-cuff recording). During the last two days, the rats were hosted in metabolic cages to measure food/energy, water, Na<sup>+</sup> ingestion, and urinary volume. Blood and kidneys were collected after euthanasia. Rostafuroxin increased food/energy and Na<sup>+</sup> intake in CTRL and RBD rats but had opposite effects on Na<sup>+</sup> balance (intake minus urinary excretion): negative in CTRL and positive in RBD. The drug normalized the decreased plasma Na<sup>+</sup> concentration found in RBD rats, increased urinary volume in RBD but not in CTRL, and decreased and increased urinary Na<sup>+</sup> concentration in the RBD and CTRL groups, respectively. Rostafuroxin decreased the ouabain-sensitive (Na<sup>+</sup>+K<sup>+</sup>)ATPase and increased the ouabain-resistant, furosemide-sensitive Na<sup>+</sup>-ATPase from proximal tubule cells in both groups, and normalized the systolic blood pressure in RBD without effect in CTRL rats. We conclude that chronic undernutrition modifies the response of blood pressure, caloric metabolism, Na<sup>+</sup> distribution in liquid compartments, mobilization of Na<sup>+</sup> from non-osmotic compartments, Na<sup>+</sup> and water balance, and activity of renal Na<sup>+</sup>-transporting ATPases to rostafuroxin.

**Keywords:** rostafuroxin; arterial hypertension; chronic undernutrition; Regional Basic Diet; sodium and water balance; renal Na<sup>+</sup>-transporting ATPases

## 1. Introduction

Rostafuroxin (a digitoxigenin derivative) [1] is an antihypertensive agent acting as an antagonist of cardiotonic steroids, including endogenous ouabain, which main molecular target is the (Na<sup>+</sup>+K<sup>+</sup>)ATPase [2]. Cardiotonic steroids are central in developing cardiovascular dysfunction,

including arterial hypertension [3]. The proposal for their existence dates back to 1885 [4,5] in a pioneering study of heart function.

Besides its classical active transporting action of maintaining the Na<sup>+</sup> and K<sup>+</sup> gradients across the plasma membrane of cells [6], growing evidence in the last two decades indicates that the ouabain-sensitive (Na<sup>+</sup>+K<sup>+</sup>)ATPase is a central signaling transducer in a great variety of physiological and pathological conditions (for excellent reviews see [7,8]). This property was why strong efforts had been developed to look for – or synthesize – compounds able to modify the interaction of the (Na<sup>+</sup>+K<sup>+</sup>)ATPase from different organs and tissues with ouabain/ouabain-like substances.

Chronic undernutrition is a pandemic growing nowadays, especially in low-income countries and impoverished areas of developed countries [9] where nearly 1 billion people are undernourished or facing a severe risk of undernutrition by eating a poor-quality diet. One widely used model of a multideficient diet for rats is that formulated based on the alimentary habits of people from several developing countries [10–13] the so-called Regional Basic Diet (RBD) [14] (Table 1) (for a recent review, see [15]). This pro-hypertensive diet [15–17] has brown beans, manioc flour, jerked meat, and sweet potatoes as the main ingredients.

**Table 1.** Composition of diets

	CTRL <sup>2</sup>	RBD <sup>3</sup>
Protein % (w/w)	23	8
Carbohydrate % (w/w)	41	78
Lipids % (w/w)	2.5	1.7
Na % (w/w)	0.3 <sup>4</sup>	0.2 <sup>4</sup>
Fe % (w/w)	0.018	0.007
Ca % (w/w)	1.8	0.04
K % (w/w)	0.9	0.3
Energy supply kcal/100 g dry weight	278	356
Vitamin supplement	Yes	No

<sup>1</sup> Reproduced from Pereira-Acácio et al. (2022) [17].

<sup>2</sup> CTRL diet. Composition indicated by the manufacturer (Neovia Nutrição e Saúde Animal, Descalvado, Brazil).

<sup>3</sup> RBD diet. Composition according to the Laboratory of Experimental and Analysis of Food (LEEAL), Nutrition Department, Federal University of Pernambuco [14].

<sup>4</sup> Determined by flame photometry (see text, section 4.6).

The current hypothesis of this study was that RBD-induced chronic undernutrition in hypertensive rats could modify the response of blood pressure, food and caloric intake, fluid and Na<sup>+</sup> handling, and active renal Na<sup>+</sup> transport to rostafuroxin.

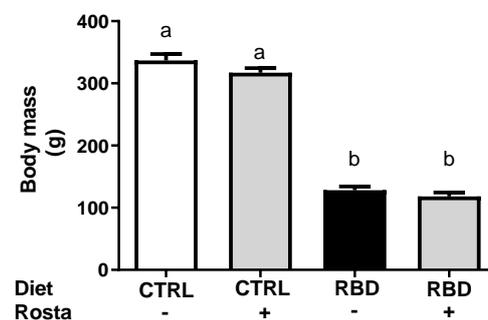
## 2. Results

### 2.1. Body Mass, Food, Energy, Na<sup>+</sup>, and Water Intake in Undernourished Rats: Effects of Rostafuroxin Administration

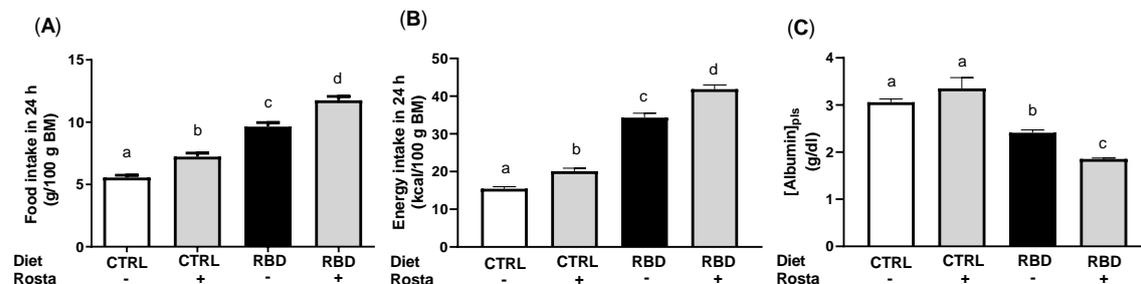
Figure 1 shows the accentuated diminution of body mass (~60%) in the group that received RBD from weaning at 28 days until 90 days of age compared to CTRL. The administration of rostafuroxin during the previous 30 days was without effect on the body mass of both groups. When food intake was evaluated at the same age (Figure 2A), we observed an increase (~70%) in RBD rats with respect

to CTRL and that rostafuroxin administration provoked an augment in both groups. The combination of higher food intake with the higher energy content of the deficient diet (Table 1) resulted in ~110% higher energy intake by RBD rats, and, also as expected from the food intake data, the rostafuroxin-treated rats incorporated much dietary energy in both CTRL and RBD rats (Figure 2B). The undernutrition of RBD rats is reflected in their decreased plasma albumin concentration ( $[\text{albumin}]_{\text{pls}}$ ) (Figure 2C) in comparison to the CTRL level. Rostafuroxin administration decreased  $[\text{albumin}]_{\text{pls}}$  in the RBD group, without influence in CTRL rats.

Despite the lower  $\text{Na}^+$  content of the RBD, the  $\text{Na}^+$  intake presented a tendency to be higher in the undernourished rats as compared with the CTRL ( $p = 0.057$ ), and the administration of rostafuroxin resulted in a significant augment of salt ingestion in the two groups (30% in CTRL and 20% in RBD; Figure 3A). The analysis of water ingestion (Figure 3B) reveals that RBD rats drank much water in the same period and that rostafuroxin had no effect in either group.



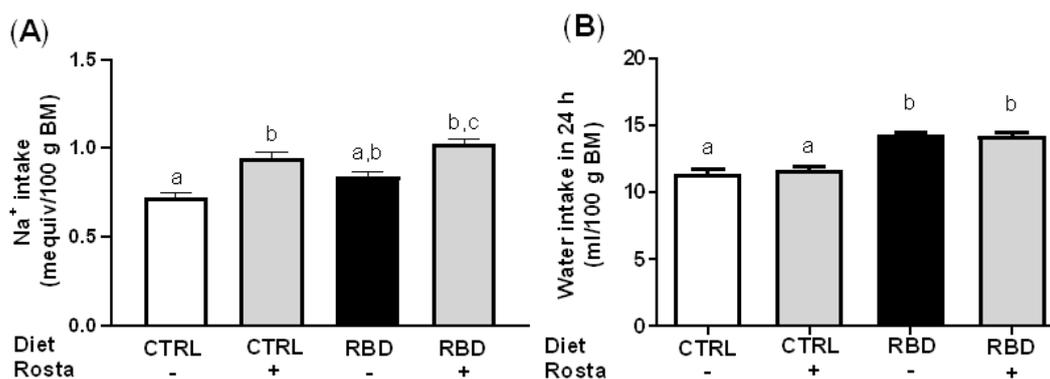
**Figure 1.** Body mass of CTRL and RBD rats at 90 days of age. Diets and treatment with rostafuroxin (Rosta) or not are indicated on the *abscissa*. Bars indicate means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 16-27$ ).



**Figure 2.** Food intake, energy intake, and plasma albumin concentration measured between 89 and 90 days of age. Diets and treatment with rostafuroxin (Rosta) or not are indicated on the *abscissae*. Bars indicate means  $\pm$  SEM. (A) Food intake:  $n = 6-9$ . (B) Energy intake:  $n = 6-9$ . (C) Plasma albumin concentration:  $n = 6-15$ . Differences were assessed using one-way ANOVA followed by Tukey's test. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ).

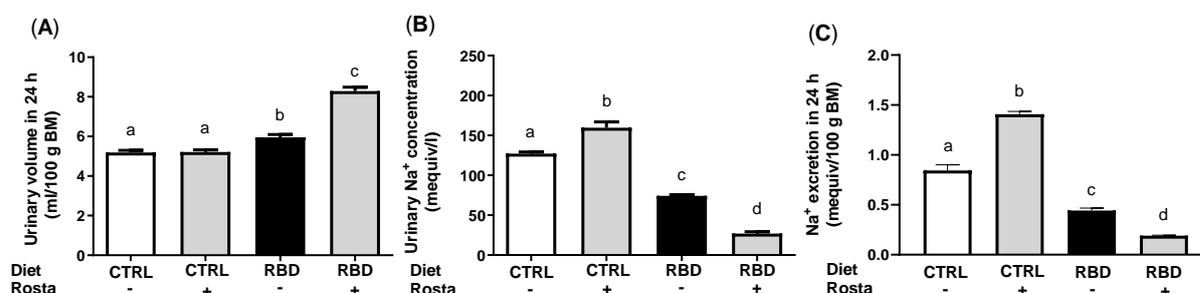
## 2.2. Urinary $\text{Na}^+$ Excretion, Urinary Volume, $\text{Na}^+$ Balance, Water Balance, and Plasma $\text{Na}^+$ Concentration in Undernourished Rats: Effects of Rostafuroxin

The urinary  $\text{Na}^+$  excretion in 24 h of rats aged 90 days was calculated from the urinary



**Figure 3.** Na<sup>+</sup> intake (A) and water intake (B) between days 89 and 90. The Na<sup>+</sup> intake in 24 h was calculated from the dietary Na<sup>+</sup> content and the food intake. Diets and treatment with rostafuroxin or not are indicated on the *abscissae*. Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 6-9$  for Na<sup>+</sup> intake;  $n = 11-15$  for water intake).

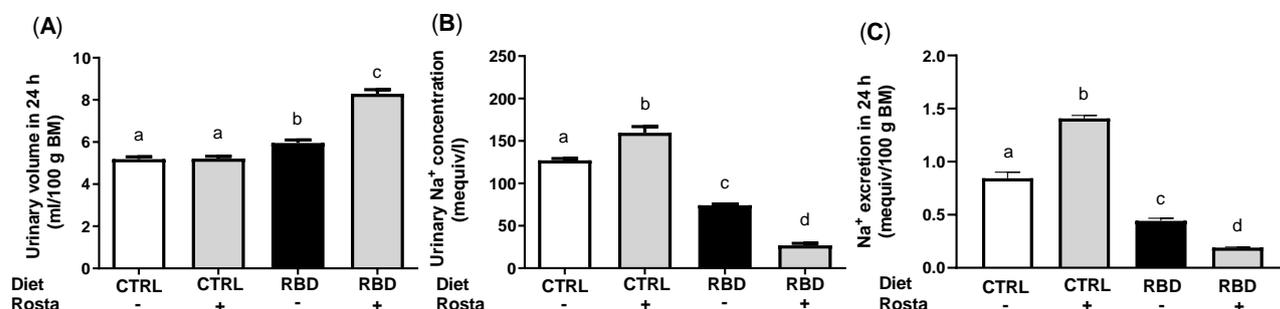
volume in the same period and the urine Na<sup>+</sup> concentration ( $[Na^+]_{ur}$ ). The urinary volume (Figure 4A) was slightly but significantly higher in RBD than in CTRL rats, and rostafuroxin provoked extra diuresis in the undernourished animals without influence in the CTRL group. The analysis of  $[Na^+]_{ur}$  (Figure 4B) revealed a 40% lower value in the RBD rats when compared to CTRL and the opposite effects of rostafuroxin depending on the nutritional status: while the drug promoted a 25% increase in  $[Na^+]_{ur}$  in normonourished rats, it provoked a diminution of near 50% in the undernourished group. The urinary Na<sup>+</sup> excretion in 24 h (Figure 4C) was 50% lower in RBD rats compared with CTRL rats. Furthermore, the influence of rostafuroxin reflects that found with  $[Na^+]_{ur}$ : a significant increase in normonourished rats and a pronounced decrease in the undernourished group.



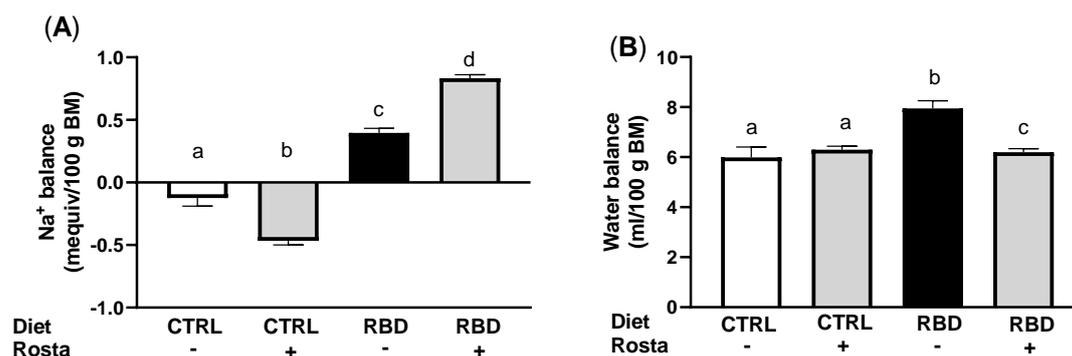
**Figure 4.** Urinary volume (A), urinary Na<sup>+</sup> concentration (B), and Na<sup>+</sup> excretion in 24 h (C) measured between days 89 and 90. Diets and treatment with rostafuroxin or not are indicated on the *abscissae*. Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 11-15$  for urinary volume;  $n = 9-15$  for urinary Na<sup>+</sup> concentration;  $n = 6-9$  for Na<sup>+</sup> excretion in 24 h).

The Na<sup>+</sup> balance at the same age, calculated as daily Na<sup>+</sup> ingestion minus urinary Na<sup>+</sup> excretion in the same 24 h-period, is seen in Figure 5A. When housed in cages, the CTRL rats presented with a slightly negative Na<sup>+</sup> balance of approximately -0.10 mequiv/100 g in 24 h, which was more negative (-0.50 mequiv/100 g in 24 h) when these animals were given rostafuroxin. In contrast, Na<sup>+</sup> balance was positive in the RBD, and the administration of rostafuroxin provoked a 100% increase. The water balance was calculated as the difference between water intake and urinary volume, and it is presented in Figure 5B. Except for the case of RBD rats (8 ml in 24 h), all animals had a daily positive water

balance of 6 ml. Concerning plasma  $\text{Na}^+$  concentration ( $[\text{Na}^+]_{\text{pls}}$ ), Figure 6 shows that it decreased to an average value of 120 mequiv/l in RBD rats from 139 mequiv/l encountered in the CTRL group. Rostafuroxin slightly, though significantly, increased  $[\text{Na}^+]_{\text{pls}}$  in normonourished and promoted a more pronounced elevation in RBD rats, reaching an average value that matched that of CTRL.



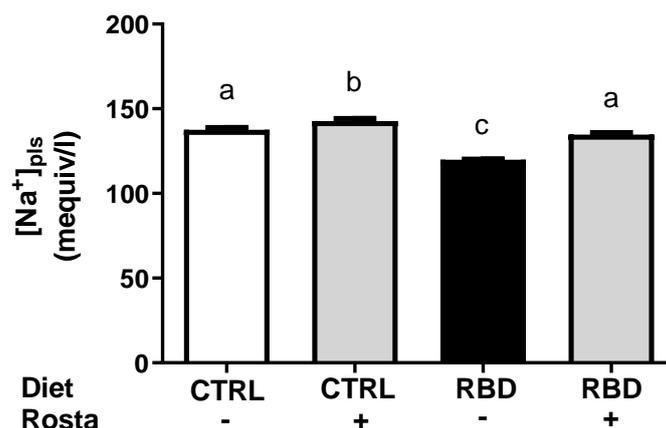
**Figure 4.** Urinary volume (A), urinary  $\text{Na}^+$  concentration (B), and  $\text{Na}^+$  excretion in 24 h (C) measured between days 89 and 90. Diets and treatment with rostauroxin or not are indicated on the abscissae. Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 11-15$  for urinary volume;  $n = 9-15$  for urinary  $\text{Na}^+$  concentration;  $n = 6-9$  for  $\text{Na}^+$  excretion in 24 h).



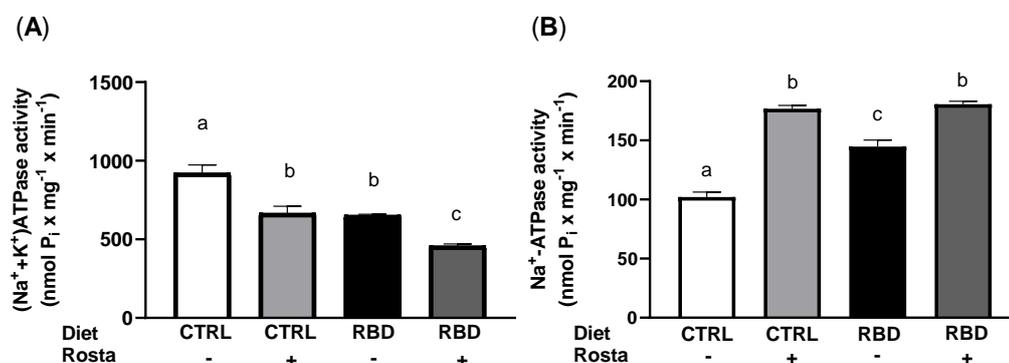
**Figure 5.**  $\text{Na}^+$  balance (A) and water balance (B). The  $\text{Na}^+$  balance was calculated as the difference between  $\text{Na}^+$  intake and urinary  $\text{Na}^+$  excretion in 24 h between days 89 and 90. The water balance was calculated as the difference between water intake and urinary volume in 24 h recorded between days 89 and 90. Diets and treatment with rostauroxin or not are indicated on the abscissae. Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 6-9$  for  $\text{Na}^+$  balance;  $n = 11-15$  for water balance).

### 2.3. $\text{Na}^+$ -transporting ATPases from Proximal Tubule Cells and Systolic Blood Pressure: Effects of Rostafuroxin

The active  $\text{Na}^+$  transport across the proximal renal tubules is a crucial process in regulating  $\text{Na}^+$  content and fluid balance in the different organs and liquid compartments [18,19]. Figure 7 shows the opposite influence of the nutritional status and rostauroxin administration on the two  $\text{Na}^+$ -transporting ATPases present in the basolateral membranes of proximal tubule cells [16]. The activity of the main pump, the ouabain-sensitive ( $\text{Na}^++\text{K}^+$ )ATPase (Figure 7A), decreased by 30% in RBD rats in comparison to CTRL, as previously described [17], with rostauroxin promoting a further decrease in both groups. In the case of the ouabain-resistant, furosemide-sensitive  $\text{Na}^+$ -ATPase (Figure 7B),



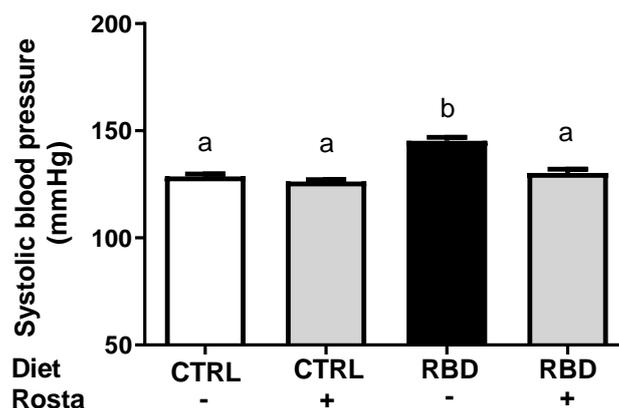
**Figure 6.** Plasma  $Na^+$  concentration ( $[Na^+]_{pls}$ ). Measurements were carried out in samples collected on day 90. Diets and treatment are indicated on the *abscissa*. Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences between means were assessed using one-way ANOVA followed by Tukey's test ( $n = 7-15$ ).



**Figure 7.** Renal proximal tubules  $Na^+$ -transporting ATPases. Determinations were carried out in plasma membrane-enriched preparations isolated from the outermost region of the renal cortex (*cortex corticis*) at day 90. Diets and treatment are indicated on the *abscissae*. (A)  $(Na^+ + K^+)ATPase$ . (B) Ouabain-resistant  $Na^+-ATPase$ . Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 4-9$  for the two activities).

undernutrition provoked a 50% increment in its activity, equalized at the same higher levels in CTRL+Rosta and RBD+Rosta after further upregulation in the animals given the drug.

Figure 8 shows that systolic blood pressure was elevated in RBD rats, which was normalized by the administration of rostafuroxin. In contrast, the drug did not modify the pressoric levels of the CTRL rats.



**Figure 8.** Systolic blood pressure. Systolic blood pressure was measured at day 90. Diets and treatment are indicated on the *abscissa*. Bars are means  $\pm$  SEM. Different lowercase letters above the bars indicate statistically different mean values ( $p < 0.05$ ). Differences were assessed using one-way ANOVA followed by Tukey's test ( $n = 3-9$ ).

### 3. Discussion

The central findings of the present study are that chronic administration of rostafuroxin – which was initially described as an antihypertensive drug with an inhibitory effect on the  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  from the kidney medulla of hypertensive rats [2] – modulates blood pressure, food/energy intake, protein metabolism, body  $\text{Na}^+$  handling, fluid balance and the  $\text{Na}^+$ -transporting ATPases resident in renal proximal tubule cells of male rats, in a way that depends on the nutritional status.

We induced chronic undernutrition from weaning until a rat juvenile age [18] by offering ad libitum a multideficient diet, RBD, which mimics those widely used in countries and regions where undernutrition is endemic [10–15]. The content of proteins is very low (Table 1), and their quality is poor because their sources are beans and jerked beef. Additionally, vitamins supplementation also does not exist. These are the reasons by which the rats body mass has markedly decreased (Figure 1), and compensatory mechanisms, likely involving neuronal circuits in the central nervous system [21–23] culminated with increased food and energy intake (Figure 2A,B). This neuronal network appears to be activated by rostafuroxin, and the stimulatory effect could involve steps in which the  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  participates [24,25] being modulated by endogenous ouabain in association with tissular  $\text{Na}^+$  levels [3,26].

At this point, it is relevant to note that rostafuroxin-treated CTRL and RBD rats consume a significantly higher amount of  $\text{Na}^+$  (Figure 3A) and, since there was an accentuated lower  $[\text{Na}^+]_{\text{plis}}$  in the undernourished group (minus  $\sim 20$  mequiv/l) (Figure 6), it could be postulated that higher  $\text{Na}^+$  is present – in a greater amount in RBD rats – in silent non-osmotic tissular compartments, which have been hypothesized some years ago [27,28] and recently revisited [29]. The results found in rats given rostafuroxin are compatible with the mobilization of  $\text{Na}^+$  from these deposits by the drug in a more accentuated way in RBD rats.

Somewhat different results regarding the potential effects of endogenous ouabain on body mass, food intake, and  $\text{Na}^+$  intake were reported more than two decades ago [30–32]. The chronic infusion of ouabain in rats increased body mass and had no effect on food or  $\text{Na}^+$  ingestion [30]; however, these observations can be explained because ouabain of external origin and endogenous ouabain seems not to be the same compounds or, in other words, that endogenous digitalis did not represent authentic ouabain [31,32]. Additionally, rostafuroxin could activate other structures beyond the central nervous system, e.g., the hypothalamic-pituitary-adrenal axis, which stimulates the secretion of glucocorticoids that augment appetite and food intake [33,34]. Other novel metabolic effects of rostafuroxin reported in the present study are those encountered in  $[\text{albumin}]_{\text{plis}}$ : decrease in RBD rats without influence in the CTRL group (Figure 2C). It could be that the drug's effect on liver protein metabolism depends on the upregulation of the AMP kinase-mediated cellular energy metabolism sensing in the liver, an organ in which AMP kinase plays a central regulatory role [35,36].

The antihypertensive effect of rostaduroxin was described as not associated with diuretic effects, despite its accentuated natriuretic action [2,37] and has not the usual side effects of diuretics in humans, as demonstrated in clinical trials [38]. We investigated whether rostaduroxin modified water balance in rats as the result of induced modifications in water intake and urinary volume and if – as in the case of food/energy intake – there was an influence of the nutrition status. The increased ingestion of water by RBD rats when compared to CTRL animals (Figure 3B) possibly represents a regulatory response facing the high ingestion of food (solutes) (Figure 2A). In contrast with that found in the case of food/energy intake, however, no rostaduroxin-induced polydipsia was found in both groups of animals, a result suggesting that hypothalamic circuits at the forebrain [39] which control thirst, have not  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  as central signaling machinery, even though the intravenous injection of ouabain decreases water intake in rats [40].

When the urinary volume in 24 h is examined, one can demonstrate that rostaduroxin had an evident diuretic influence only in RBD but not in CTRL rats (Figure 4A). This observation allows us to conclude that, again, the nutritional status of the animals influences one important effect of endogenous ouabain. Since the pioneering studies mentioned above were carried out with normonourished rats [2,37], the lack of diuretic action may only occur in properly nourished animals. This idea receives support from the observation that neither urinary volume nor water intake was modified by rostaduroxin administration in CTRL rats (Figures 4A and 3B). The diuretic effect of rostaduroxin in RBD rats led to the normalization of water balance to CTRL levels (Figure 5B); only the untreated RBD continued with a positive water balance (Figure 5B), possibly as the result of the presence of elevated levels of circulating endogenous ouabain, which has been implicated in water retention [30].

Rostaduroxin-induced natriuresis was one of the more noticeable effects described early [2,37]. This action was attributed to a counteracting influence on circulating endogenous ouabain on renal  $(\text{Na}^+\text{+K}^+)\text{ATPase}$ , principally that localized in the external medulla [2], because of the role of the  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  resident in the basolateral membranes of the thick ascending segment of Henle loop in the final control of  $\text{Na}^+$  excretion in physiological and pathological conditions [41]. This picture was confirmed in the case of CTRL rats, which presented with increased  $[\text{Na}^+]_{\text{ur}}$  and  $\text{Na}^+$  excretion in 24 h when submitted to a chronic administration of rostaduroxin. However, a contrasting effect was encountered in RBD rats: their diminished  $[\text{Na}^+]_{\text{ur}}$  and  $\text{Na}^+$  excretion in 24 h was further decreased by rostaduroxin (Figure 4B,C). As proposed above, the undernourished rats may present with augmented endogenous ouabain production in the central nervous system that could cause increased nerve renal activity, thus eliciting an antinatriuretic response [42]. In this condition, abnormal activation of the renal nerve could be the underlying basis of the opposite, strong antinatriuretic influence of rostaduroxin, which actions are dose-dependent and also dependent on the ouabain levels when this drug is infused [43].

The results regarding  $[\text{Na}^+]_{\text{ur}}$  and  $\text{Na}^+$  excretion in 24 h presented in Figure 4B,C reverberate in the bodily  $\text{Na}^+$  balance depicted in Figure 5A where, in which, as expected, the influence of nutritional status clearly appears. The untreated CTRL rats had an overall  $\text{Na}^+$  balance that approached zero, as usually occurs with part of the rat population hosted in metabolic cages for a short period [44]. When rostaduroxin was given to these animals, a significantly negative daily balance ( $\sim 0.6$  mequiv  $\text{Na}^+$  per 100 g BM in 24 h) was found, despite the unmodified  $\text{Na}^+$  intake (Figure 3A). This trend also suggests that rostaduroxin mobilizes the ion from a so-called non-osmotically active “spooky” compartment [45], transiently passing through a plasma compartment slightly concentrated with  $\text{Na}^+$  as seen in Figure 6. If we apply this reasoning to RBD rats, the daily positive balance means a cumulative  $\text{Na}^+$  storage along the experimental period of the assay (62 days). This positive balance doubled in the rats that received rostaduroxin, likely due to an abnormal renal response to the drug in a condition of probably elevated endogenous ouabain, mimicking the experimental antinatriuretic conditions established by Lim et al. [42].

In a recent study [17] and here (Figure 7A,B) we demonstrated that chronic undernutrition provoked by the continued administration of RBD to male rats downregulates the ouabain-sensitive  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  and upregulates the ouabain-resistant  $\text{Na}^+\text{-ATPase}$ , the two  $\text{Na}^+\text{-transporting}$  ATPases that are responsible for the proximal renal reabsorption of more than 70% of the  $\text{Na}^+$  filtered in the glomeruli [46,47]. Even though the hydrolytic activity of  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  in membrane

preparations does not quantitatively reflect the transport stoichiometry in intact cells, it is possible to propose that less bulk  $\text{Na}^+$  is reabsorbed in these tubules in undernourished rats because the lower transport demand in lower-sized animals. Conversely, upregulation of the one order of magnitude lower ouabain-resistant ATPase means that the fine-tuned  $\text{Na}^+$  reabsorption across the proximal epithelium mediated by this enzyme is increased, possibly contributing to the progressive genesis of arterial hypertension [48]. Concerning rostafuroxin, the influence of the drug on  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  (Figure 7A) matches the  $\text{Na}^+$  balance in CTRL rats, and also that effect of the drug on the medullary  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  [2]. In the case of RBD rats, there is a total dissociation between  $\text{Na}^+$  balance and  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  in both untreated and untreated animals (compare Figure 5A and Figure 7A). The results with undernourished rats are compatible with the idea that migration of the  $(\text{Na}^+\text{+K}^+)\text{ATPase}$  from the membrane and abnormal anchoring to cytoskeletal proteins has occurred, thus downregulating this pump [32]. The opposite is true when we look at the ouabain-resistant  $\text{Na}^+\text{-ATPase}$  (Figure 7B), which its influence on the  $\text{Na}^+$  balance – despite its importance in body  $\text{Na}^+$  handling – is not evident due to its lower activity (compare ordinates in Figure 7A,B).

Finally, one last question deserves discussion: is there any diet-modulated effect of rostafuroxin on the systolic blood pressure encountered in undernourished rats? Figure 8 shows that the drug completely normalized the arterial pressure in the RBD group without influence in CTRL rats and these facts could be discussed on the basis of three ideas: (i) the structural and functional undernutrition-induced abnormalities proposed to occur in renal tissue on the basis of the Lim et al. [42] hypothesis above discussed – which alter the responses of the two  $\text{Na}^+$ -transporting ATPases to rostafuroxin (Figure 7A,B) – are not present in arteries such as thoracic aorta and mesenteric arteries; (ii) rostafuroxin seems to act on these vessels only when a “pro hypertensive environment” has developed [49] such as described in hypertensive rats [2,49,50, and this study]; (iii) removal of silent  $\text{Na}^+$  promoted by rostafuroxin from the water-free stores represented by glycosaminoglycans from the endothelial surface and from the perivascular interstitium [51,52] could also underpin the antihypertensive effect of rostafuroxin in undernourished rats.

In one type of spontaneously hypertensive rats with high levels of endogenous ouabain, rostafuroxin did not interact with the renin-angiotensin-aldosterone system (RAAS) [2] and, therefore, the antihypertensive actions of the drug seem not to antagonize RAAS to prevent the genesis of hypertension. In the case of RBD undernourished rats, the cortical renal tubulointerstitium has more than four times the number of angiotensin II-positive cells found in CTRL rats [53], evidence of an increased tissular RAAS activity. Moreover, rostafuroxin-sensitive hypertension they develop (Figure 8) is also completely normalized by the administration of losartan, an antagonist of type 1 angiotensin II receptors [17]. In terms of vascular actions in vessels (such as the thoracic aorta and mesenteric arteries), rostafuroxin appears to have opposite effects and the same final targets as RAAS. It has been proposed that endogenous ouabain is regulated by type 2 angiotensin II receptors [3,54,55] which initiate the branch of RAAS that antagonizes the type 1 angiotensin II pathway [56]. Thus, the cross-talk between rostafuroxin-associated signaling pathways and RAAS deserves further studies.

## 4. Materials and Methods

### 4.1. Ethical Statement

The experimental procedures were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Rio de Janeiro (protocol 012/19). They were performed following the Committee’s Guidelines, which follow the Uniform Requirements for Manuscripts Submitted to Biomedical Journals published by the International Committee of Medical Journal Editors. The animal study is reported following ARRIVE guidelines [57].

### 4.2. Diets

The Regional Basic Diet (RBD) is a model of a multideficient diet [14] that mimics the alimentary habits of vast, impoverished regions of Northeast Brazil and worldwide [10–14]; its composition is given in Table 1. RBD was prepared under bromatological control in a homemade process using the

facilities of the Laboratory of Food Analysis and Processing of the Josué de Castro Institute of Nutrition at the Federal University of Rio de Janeiro. The ingredients were cooked, oven-dried at 60 °C, and ground before mixing. Water was added to form a sticky wet mass cut in small pieces – as the standard commercial control diet (CTRL) – and finally dehydrated for one day at 60 °C. The composition of the CTRL diet (Neovia Nutrição e Saúde Animal, Descalvado, Brazil) (Table 1) follows the recommendations of the American Institute of Nutrition for rodents (AIN-93G) [58]. The dietary Na<sup>+</sup> content was determined by flame photometry after acid extraction with 1 N HNO<sub>3</sub>. The minerals (K<sup>+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>) and vitamin content were those encountered after mixing the dietary components, as previously determined [14,59–61].

#### 4.3. Experimental Groups

Female Wistar rats ( $n = 20$ ) were kept and mated (4 females:1 male) in the Vivarium of Neglected Diseases and Undernutrition of the Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro. Male offspring were weaned at 28 days of age. The animals were randomly divided into two groups: one received the RBD *ad libitum*, and the other received the CTRL diet until they reached 90 days of age. Drinking water was also offered *ad libitum*. When the rats reached 60 days, the two groups were randomly subdivided into two other subgroups, originating those that received Rostafuroxin (Rosta: 1 mg/kg body mass diluted in 99% ethanol; Aobious Inc, Gloucester, MA, USA) or the same volume of water. Thus, the four experimental groups were: CTRL, CTRL+Rosta, RBD, and RBD+Rosta. At 89 days of age, the rats were placed in metabolic cages to measure water ingestion, food ingestion, and urinary volume. After the metabolic cage period (1 day), the blood pressure was measured. Finally, the rats were euthanized by decapitation, the blood was collected under EDTA, and the plasma was immediately separated to measure the plasma Na<sup>+</sup> concentration ( $[Na^+]_{pls}$ ). The kidneys were carefully dissected to obtain plasma membrane preparations from proximal tubule cells.

#### 4.4. Measurement of Blood Pressure

Systolic blood pressure was measured by tail-cuff plethysmography [62] in conscious rats immediately after the cage period (day 90) by using the Insight system model V2.01 (Bonther, Ribeirão Preto, Brazil), coupled to the appropriate software (Pressure Gauge 2.0, Insight). The rats were acclimated in a heated chamber (30–32 °C) for 10 to 15 min, and the recordings were only taken when the rats did not present sudden movements. Five determinations were made for each rat, and the average of the five values was used.

#### 4.5. Preparation of Plasma Membrane-enriched Fraction from Kidney Proximal Tubule Cells

After the removal of the kidneys, the membrane preparations were obtained by homogenization and differential centrifugations of tissue fragments from the outer region of the renal cortex (*cortex corticis*) [63], where the cell population corresponds to >95% of proximal tubules. The preparation procedure was as described by Silva *et al.* [16]. Protein content was determined using the method described by Lowry *et al.* [64]. The samples were used to measure the activity of the two Na<sup>+</sup>-transporting ATPases, the ouabain-sensitive (Na<sup>+</sup>+K<sup>+</sup>)ATPase and the ouabain-resistant, furosemide-sensitive Na<sup>+</sup>-ATPase [16].

#### 4.6. Determination of Na<sup>+</sup> in Urine and Plasma Samples

Na<sup>+</sup> concentrations were determined by flame photometry (Analyzer, São Paulo, Brazil) using a standard solution containing 140 mequiv Na<sup>+</sup>/l (Analyzer). The urinary Na<sup>+</sup> excretion in 24 h was calculated using the urinary volume in this period and urinary Na<sup>+</sup> concentration. The Na<sup>+</sup> balance was calculated as the difference between Na<sup>+</sup> intake and Na<sup>+</sup> excretion in the same 24-period. The Na<sup>+</sup> intake was calculated from the ingestion of food and the dietary Na<sup>+</sup> content (Table 1).

#### 4.7. Determination of Albumin in Plasma Samples

The plasma albumin concentration was measured using a commercial kit (Bioclin, Belo Horizonte, Brazil).

#### 4.8. Determination of the Activities of Na<sup>+</sup>-transporting ATPases

The ouabain-sensitive (Na<sup>+</sup>+K<sup>+</sup>)ATPase and the ouabain-resistant, furosemide-sensitive Na<sup>+</sup>-ATPase activities were determined by quantifying the inorganic phosphate (P<sub>i</sub>) released during ATP hydrolysis [65]. The activities were determined in triplicate precisely as described by Silva *et al.* [16]. The activities were calculated by the differences between the values obtained in the absence and presence of 2 mM ouabain (for (Na<sup>+</sup>+K<sup>+</sup>)ATPase); and between the values obtained in the absence and presence of 2 mM furosemide, always in the presence of 2 mM ouabain (for Na<sup>+</sup>-ATPase).

#### 4.9. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 8 software (version 8.01, GraphPad Software, Inc., San Diego, CA, USA). Results are expressed as means ± SEM. The analysis was one-way ANOVA followed by Tukey's test, as indicated in the figure legends. Significant differences were set at  $p < 0.05$ .

### 5. Conclusions

In conclusion, we demonstrated that rostaduroxin exerts antihypertensive effects in undernourished hypertensive rats without influence in normonourished normotensive rats. Moreover, the present study also provides evidence demonstrating that undernutrition provoked by a low-protein, low-salt, hypercaloric diet modifies the response of caloric and albumin metabolism, Na<sup>+</sup> distribution, Na<sup>+</sup> and water balance, and the activity of renal Na<sup>+</sup>-transporting ATPases to the drug. Although there is also evidence regarding the involvement of endogenous ouabain and alterations of a central nervous system → renal axis, the molecular mechanisms of this connection are still unknown.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee for the Use of Animals in Scientific Experimentation of Federal University of Rio de Janeiro, Brazil (protocol code 12/019; approved April 30, 2019).

**Data Availability Statement:** In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to

suggested Data Availability Statements in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. You might choose to exclude this statement if the study did not report any data.

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