

**Undernutrition Fetal Programming: Effects on Kidney Morphology,
Renal Steroid and Angiotensin Receptors Expression and Urinary
Sodium Excretion**

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Palavras-chave: Fetal programming; Hypertension; Renal function; Rennin-angiotensin system; Undernutrition.

ABSTRACT

Background. Maternal undernutrition affects the foetal development, promoting renal alterations and adult hypertension. The present study investigates, in adult male rats, the effect of food restriction *in utero* on arterial blood pressure changes (AP), and its possible association with the number of nephrons, renal function and angiotensin II (AT_{1R}/AT_{2R}), glucocorticoid (GR) and mineralocorticoid (MCR) receptors expression.

Methods. The daily food supply to pregnant rats was measured and one group (n=5) received normal quantity of food (NF) while the other group received 50% of that (FR50) (n=5). The AP was measured weekly. At 16 weeks of life, fractionator's method was used to estimate glomeruli number in histological slices. The renal function was estimate by creatinine and lithium clearances. Blood and urine samples were collected to biochemical determination of creatinine, sodium, potassium and lithium. At 90th and 23rd days of life, kidneys were also processed to AT_{1R}, AT_{2R}, GR and MCR immunolocalization and for western blotting analysis.

Results. FR50 offspring shows a significant reduction in BW (FR50: 5.67 ± 0.16 vs. 6.84 ± 0.13 g in NF, $P < 0.001$) and increased AP from 6th to 12nd week (6thwk FR50: 149.1 ± 3.4 vs. 125.1 ± 3.2 mmHg in NF, $P < 0.001$ and, 12ndwk FR50: 164.4 ± 4.9 vs. 144.0 ± 3.3 mmHg in NF, $P = 0.02$). Expression of AT_{1R} and AT_{2R} were significantly decreased in FR50 (AT₁, 59080 ± 2709 vs. 77000 ± 3591 in NF, $P = 0.05$; AT₂, 27500 ± 95.50 vs. 67870 ± 1509 in NF, $P = 0.001$) while the expression of GR increased in FR50 (36090 ± 781.5 vs. 4446 ± 364.5 in NF, $P = 0.0007$). The expression of MCR did not change significantly. We also verified a pronounced decrease in fractional urinary sodium excretion in FR50 offspring (0.03 ± 0.02 vs. 0.06 ± 0.04 in NF, $p = 0.03$). This occurred despite unchanged creatinine clearance.

Conclusion. The study led us to suggest that fetal undernutrition, with increased fetal exposure to maternal corticosteroids, *program* to persistent renal glucocorticoid receptor upregulation in adulthood life. That effect may be related to development of hypertension in progenies. Additionally, downregulation of the angiotensin II receptors may result in lack of ability of renal tubules water and salt handling, which in turn may also contribute to hypertension establishment.

Keywords: fetal programming; hypertension; renal function; rennin-angiotensin system; undernutrition

INTRODUCTION

Fetal programming by maternal malnutrition results in low birth weight and reduction in nephron number [1-3] increasing the risk for adult development of cardiovascular and renal diseases [4,5]. Recent studies have displayed that low birth weight in humans increased in 70% the risk for end-stage kidney failure in adults [6]. Experiments have been shown that renal programming with nephron number reduction may occur when birth weight is normal [7,8]. It is important to note that the consequences of fetal programming are not limited to the first generation and these effects may be prolonged to subsequent generations [9]. The nephron number reduction and hypertension provoked by gestational protein restriction are transmitted for F1 and F2 generations from both males and females programmed [10]. The renal programming has been studied in a big number of animal models and recently we showed that rats submitted to gestational low protein diet presented 8% lower birth weight and hypertension from 10th week of life parallels with 30% reduced nephron number. With 16 week-old, the renal Na⁺ excretion was 50% lower and angiotensin II receptors were reduced [11]. Different animal models and gestational diets or insults may result in distinct programming effects and also different compensative challenges to maintaining homeostasis from birth to adult life. The renin-angiotensin-system (RAS) has been implicated in the major renal programming studies [12]. The more discussed mechanism involved in fetal programming is the reduction in expression and/or activity of placental 11 β HSD-2 exposing fetus to high concentration of maternal glucocorticoids [13,14]. The action of maternal corticoid in mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) receptors may have impact in renal development [15,16]. In this way we suppose that after birth the day-to-day adaptations of programmed rats in way of homeostasis maintenance can involve alterations in expression/localization of these receptors. Recently, it has been speculated that maternal control, correcting down-regulation of placental amino acid transporters may be involved in fetal programming by gestational protein restriction [17]. The purpose of the present study was to determine whether maternal undernutrition during whole pregnancy alters kidney expression of angiotensin II (AT1 and AT2), mineralocorticoid (MR) and glucocorticoid (GR) receptors in male offspring rats. Since we have verified in protein restriction model that the long-term changes in renal sodium tubule handling is associated with arterial hypertension development, we also hypothesized that hypertension caused by malnutrition may result from decreased

urinary sodium excretion in the offspring. For comparative analysis of these two models, we studied the tubular sodium handling, evaluated by lithium clearance, in conscious maternal undernourished rats compared with and their appropriate normal maternal food intake controls.

MATERIAL AND METHODS

Animals - The experiments were conducted on age-matched, female offspring of sibling-mated Wistar Hannover rats (0,250-0,300 kg) allowed free access to water and normal rat chow. The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the investigation. Our local colonies originated from a breeding stock supplied by CEMIB/Unicamp, Campinas, SP, Brazil. Immediately after weaning at 3 weeks of age, animals were maintained under controlled temperature (25°C) and lighting conditions (7:00 a.m. – 7:00 p.m.), with free access to tap water and standard rodent laboratory chow (Nuvital, Curitiba, PR, Brazil) and followed up to 12 weeks of age. Animals were then mated and the day that sperm were seen in the vaginal smear was designated as day 1 of pregnancy. The dams were divided into two groups: throughout entire pregnancy, the daily food supply of one group (FR50) was restricted to 50% of the food consumed by the other group (NF), fed *ad libitum*. All groups returned to the *ad libitum* chow intake after delivery. Body weight and food consumption was determined every day (subsequently normalized for body weight). The male pups were weighted, followed and maintained with normal chow *ad libitum* until adulthood.

Blood Pressure Measurement - The systemic arterial pressure was measured in conscious 6, 8, 9, 10, 11 and 12-week-old rats by an indirect tail-cuff method using an electrosphygmomanometer (Narco Bio-Systems, Austin, TX) combined with a pneumatic pulse transducer/amplifier. This indirect approach allowed repeated measurements with a close correlation (correlation coefficient = 0.975) compared to direct intra-arterial recording. The mean of three consecutive readings represented the blood pressure.

Renal Stereology – 16 weeks-old male rats from the NF (n=4) and FR50 (n=4) groups were used. The rat was killed and the kidney removed, weighted and the volume was estimated by Cavalieri's principle. Kidneys were embedded face down then serially sectioned at a nominal thickness of 5 μ m, being stained with hematoxylin and eosin. Fractionator method was used to estimate glomeruli number in a slice. The total number of glomeruli per kidney was estimated considering the analyzed fraction of the kidney corrected to the entire organ.

Renal function tests - The renal function tests were performed on the last day at 16 weeks of age in unanaesthetized, unrestrained NF ($n = 17$) and RF50 ($n = 11$) male rats. Creatinine and lithium clearance were done as standard methodology [18].

Calculations and biochemical determinations - Plasma and urinary sodium, potassium and lithium concentrations were measured by flame photometry (B262; Micronal, São Paulo, Brazil). Creatinine was determined spectrophotometrically (362; Micronal, São Paulo, Brazil) by the alkaline picrate method. The results are reported as means $\pm$ SEM per 100 g body weight. Renal clearance was calculated by a standard formula ($C = UV/P$) using the plasma creatinine and lithium levels for each period. C_{Cr} was used to estimate the glomerular filtration rate and C_{Li+} was used to assess proximal tubule output. FE_{Na+} (fractional urinary sodium excretion) and FE_{K+} (fractional potassium excretion) were calculated as C_{Na+}/C_{Cr} and C_{K+}/C_{Cr} , respectively. FEP_{Na+} (fractional proximal sodium excretion) and $FEPP_{Na+}$ (fractional post-proximal sodium excretion) were calculated as $C_{Li+}/C_{Cr} \times 100$ and $C_{Na+}/C_{Li+} \times 100$, respectively [18-20].

Tissue extracts – 23 and 90 days-old male rats from the NF (n=5) and FR50 (n=5) groups were used. The animal's abdominal cavity was opened to kidney removal. The tissue was minced coarsely and homogenized immediately in 10 volumes of solubilization buffer [10 ml/L Triton-X 100, 100 mmol/L Tris[hydroxymethyl]amino-methane (Tris) pH 7.4, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L ethylenediaminetetracetic acid (EDTA), 10 mmol/L sodium vanadate, 2 mmol phenylmethylsulfonyl fluoride (PSMF) and 0.1 mg/ml aprotinin] at 4°C, using a polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, N.Y., USA) operated at maximum speed for 20 s. The tissue extracts were centrifuged at 11.000 rpm at 4°C for 40 min, and the supernatants used as sample.

Antibodies and chemicals- Protein quantification was performed using the Bradford method. For quantification, both tissue and total extract samples (250µg protein) were subjected to SDS-PAGE. After electrophoretic separation, proteins were transferred to nitrocellulose membranes and then blotted with specific antibody. The samples were treated with Laemmli buffer containing 100 mmol/l dithiothreitol (DTT), heated in a boiling water bath for 4 min and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad minigel apparatus (Mini-Protean, Bio-Rad). Electrotransfer of proteins from the gel to the nitrocellulose membranes was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin *et al.*, (1979) [21]. The non-specific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at 22°C in blocking buffer (5% non-fat dry milk, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20). The nitrocellulose blots were incubated at 4°C overnight with primary antibodies diluted in blocking buffer (3% non-fat dry milk, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20). Immunoreactive bands were detected using the enhanced chemiluminescence method (RPN 2108 ECL Western blotting analysis system; Amersham Biosciences) and were detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screen (DuPont, Wilmington, DE) for 10 min. Images of the developed radiographs were scanned (Epson Stylus 3500) and band intensities were quantified by optical densitometry (Scion Image Corporation).

Tissue processing, histology and immunohistochemical procedures- 23 days-old and 16 weeks-old male rats from the NF (n=5) and RF50 (n=5) groups were anesthetized with a mixture of ketamine (75 mg .kg-1body weight, i.p.) and xylazine (10mg.kg-1body weight, i.p.) and the level of anesthesia was controlled by monitoring the corneal reflex. The animals were then perfused with saline containing heparin (5%) for 15 min under constant pressure, followed by perfusion with 0.1M phosphate buffer (pH 7.4) containing 4% (w/v) paraformaldehyde and 0.1 mol/L (M) sucrose for 25 min. After perfusion, kidneys were removed, weighted, and representative samples were fixed in 4% phosphate-buffered formalin during 24 h for paraffin embedding. For immunohistochemical analysis we use anti-AT1, AT2, MCR and GR antibodies.

Proteins expression was immunohistochemically detected using the avidin–biotin–peroxidase method. Briefly, deparaffinized 5- μ m-thick heart sections on poly-l-lysine coated slides were treated with 3% H₂O₂ in phosphate-buffered saline for 15 min, nonfat milk for 60 min, primary antibodies for 60 min, and avidin–biotin–peroxidase solution (Vector Laboratories Inc, CA, USA, 1:1:50 dilution). Antigen retrieval was performed using 0.01 M citrate buffer (pH 6.0) boiling in microwave oven (1,300 W) twice for 5 min each. Chromogen color was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich Co., St. Louis MO, USA) as the substrate to demonstrate the sites of peroxidase binding. The slides were counterstained with Harris's hematoxylin.

Statistical analysis - All data were reported as means $\pm$ SEM. Comparisons involving only two means within or between groups were done using Student's *t*-test. A *P*-value < 0.05 was considered to indicate significance.

RESULTS

FR50 offspring presented significant reduction in birth body weight (5.67 ± 0.16 vs 6.84 ± 0.13 in NF, $p < 0.001$). This reduction remained in the offspring at 16 weeks of life (385.5 ± 23.02 vs 448.5 ± 10.87 in NF, $p = 0.048$) (Figure 1).

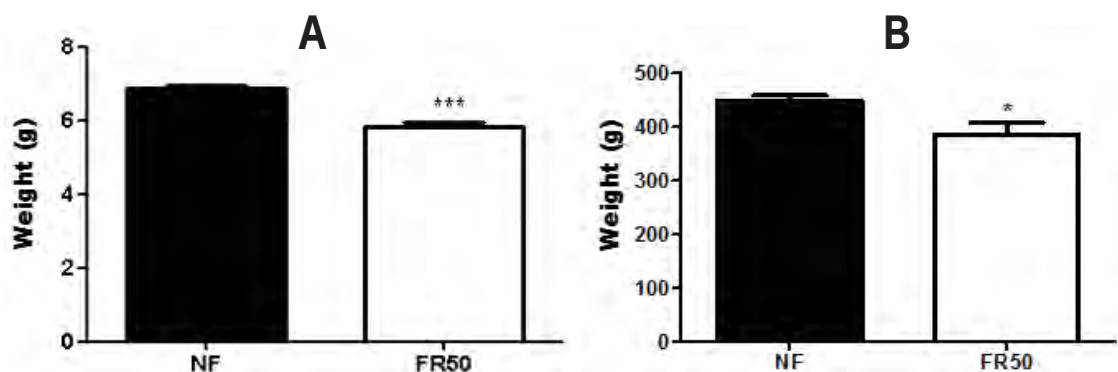


Fig. 1. Effects of maternal undernutrition in the weight of the offspring. **A:** at birth. **B:** with 16 weeks of life. The data are reported as the means $\pm$ SEM. * $P \leq 0.05$ and *** $P \leq 0.001$ versus control (Student's *t*-test).

Systolic blood pressure increased in FR50 (Figure 2) from 6th to 12nd week (6th, 149.1 ± 3.4 v 125.1 ± 3.2 in NF, $p < 0.001$; 12nd, 164.4 ± 4.9 v 144.0 ± 3.3 in NF, $p = 0.02$). By stereological analyses we verified that FR50 offspring presented reduction

of nephron numbers per kidney, although this value has not been significant ($p=0.11$). The overall size of the kidney did not change in this model.

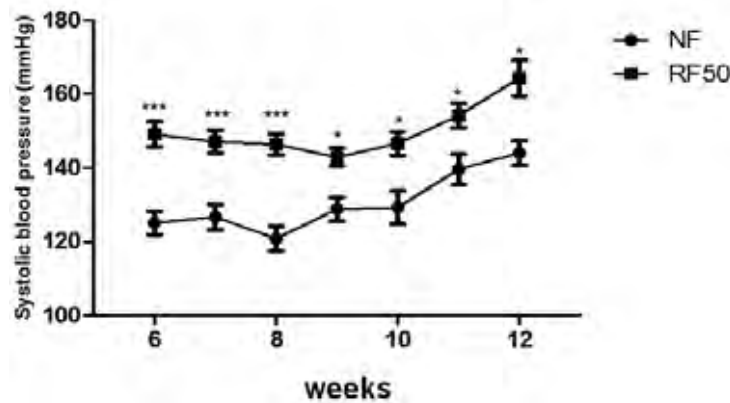


Figure 2. Systolic blood pressure in NF and FR50 animals from 6th to 12nd week of age. The data are reported as the means $\pm$ SEM. * $P \leq 0.05$ and *** $P \leq 0.001$ versus control (Student's t-test).

Renal function data

The data for renal function in the 16-week-old offspring of both (NF and FR50) groups are summarized in Figure 3.

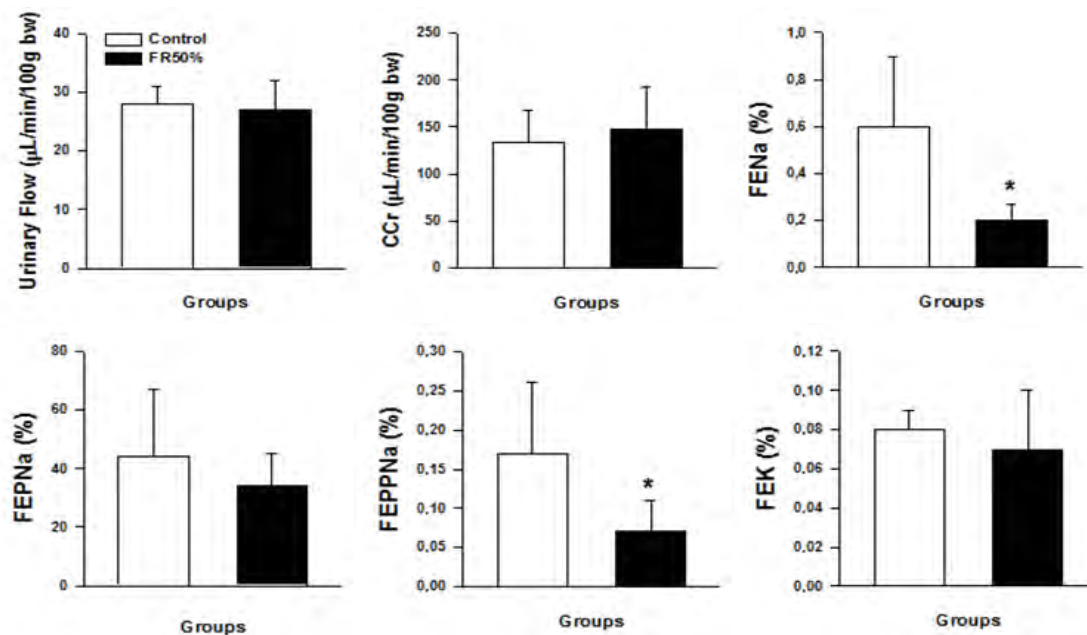


Fig 3. Renal function. Creatinine clearance (C_{Cr}), fractional sodium excretion (FE_{Na}), proximal (FEP_{Na}) and post-proximal ($FEPP_{Na}$) fractional sodium excretion and fractional potassium excretion (FE_K) in NF and FR50 (16-week-old offspring). The data are reported as the means $\pm$ SEM. * $P \leq 0.05$ versus control (Student's t-test).

The urinary flow rates and the glomerular filtration rate, estimated by CCr , did not significantly differ among the groups during the renal tubule sodium handling studies (147 ± 40 vs. 136 ± 27 in NF, $p=0.39$). FE_{Na+} was significantly lower in FR50

rats when compared with NF age-matched group, as follows: 0.03 ± 0.02 vs. 0.06 ± 0.04 in NF ($p=0.03$). The decreased FE_{Na+} in FR50 rats was accompanied by unchanged proximal sodium excretion (FEP_{Na+}) and fractional potassium excretion (FE_{K+}) compared with the NF age-paired control group. This decreased FE_{Na+} occurred in parallel to significant reduction in post-proximal sodium excretion $FEPP_{Na+}$ in the FR50.

Western blot analysis

Western blot analysis in male offspring of NF and FR50 rat kidneys yielded a single band at the expected weight of corresponding proteins. The MCR expression in 23 and 90 days old rats was not statistically different between NF and FR50 (23 days: 873.30 ± 21.97 vs. 642.60 ± 51.65 in NF; 90 days: 1151.00 ± 41.98 vs. 987.70 ± 70.20 in NF). The expression of GR increased in FR50 but this rise was significant only in 90 days old rats (23 days: 507.00 ± 91.02 vs. 262.60 ± 56.16 in NF, $p=0.15$; 90 days: 444.60 ± 36.45 vs. 360.0 ± 78.15 in NF, $p=0.0007$), how showed in figure 4. The expression of $AT1_R$ was reduced in FR50 being significant only in 90 days old rats (23 days: 702.10 ± 46.01 vs. 799.30 ± 18.44 in NF, $p=0.19$; 90 days: 590.80 ± 27.09 vs. 770.00 ± 35.91 in NF, $p=0.05$). $AT2_R$ expression was significantly decreased in 23 and 90 days old rats from FR50 group (23 days: 580 ± 23 vs. 786 ± 91 in NF, $p=0.01$; 90 days: 275 ± 95.5 vs. 678.7 ± 15 in NF, $p=0.001$).

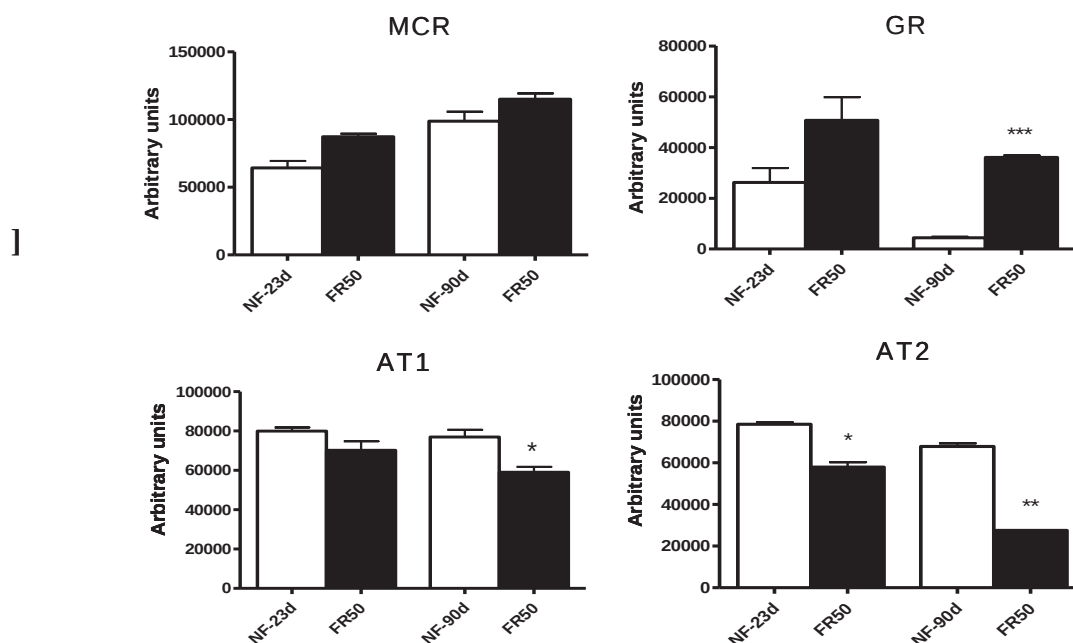


Fig. 4. Effects of maternal malnutrition on expression of $AT1_R$, $AT2_R$, GR and MCR in the kidneys from NF e FR50 rats with 23 and 90 days-old. The results of scanning

densitometry were expressed as relative to NF. Columns and bars represent the mean $\pm$ SEM. *P < 0.05, **P < 0.01 and ***P < 0.001, NF versus FR50.

Immunohistochemical analysis

Although by western blot we have not found significant differences in the expression of MCR in 23 and 90 days old rats, by immunohistochemistry we verified increased immunoreactivity of this receptor in the basolateral membrane of the cells of the proximal convoluted tubules in animals of FR50 group (figure 5). In rats with 16 weeks, we found increased expression of MCR in the cytosol of glomerular cells and in parietal epithelium of Bowman's capsule (Figure 6 - A and E). This increase was also observed in the nuclei and cytosol of post-proximal segments of both cortex (Figure 6 - B and F) and medulla (Figure 6 - D and H). In the proximal segments the immunoreactivity was enhanced in the brush border (Figure 6 - C and G).

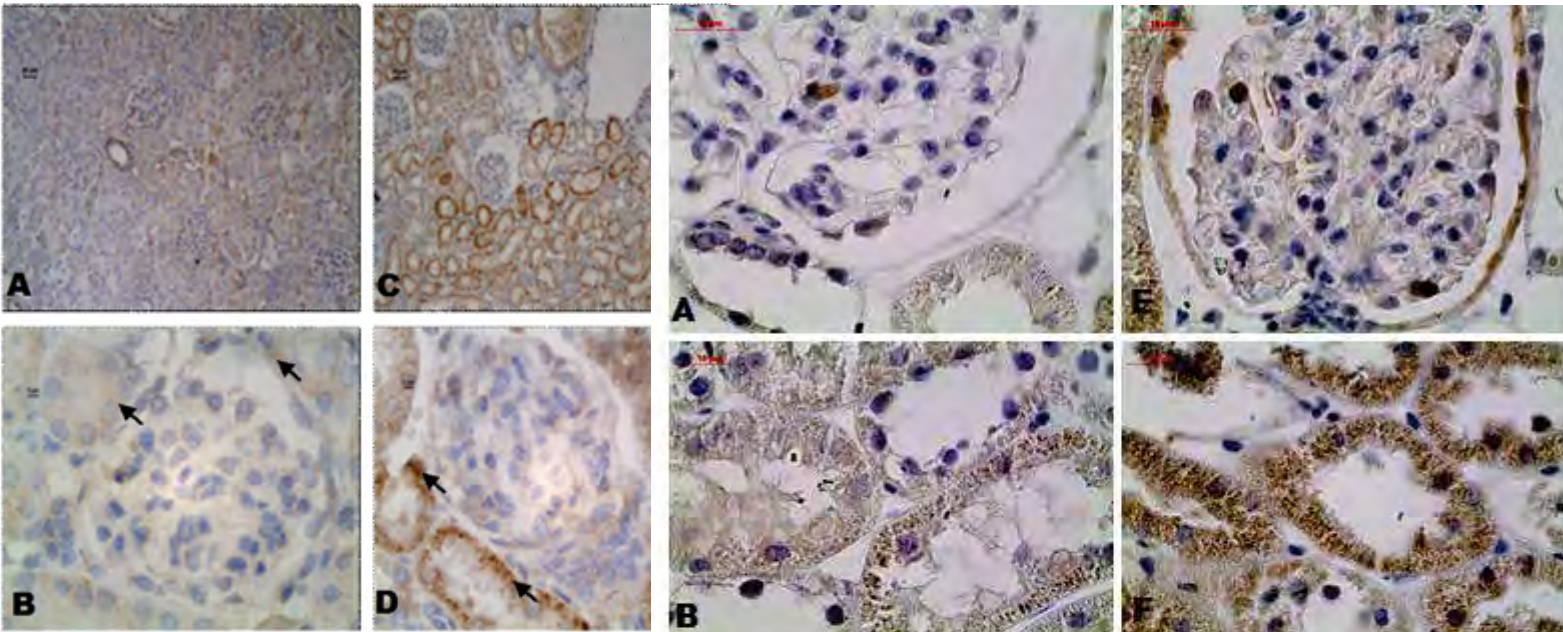
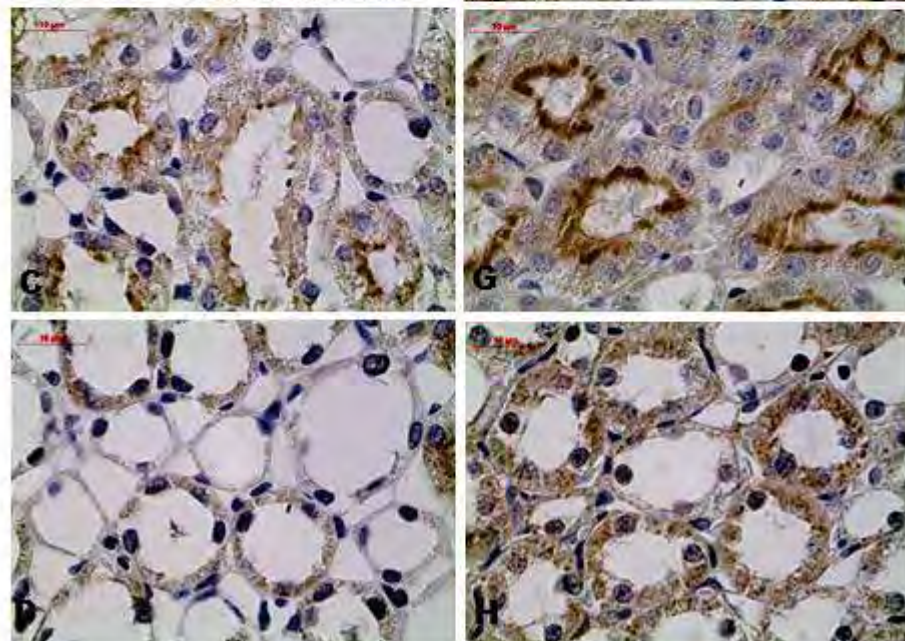


Fig. 5. (above) MCR kidney expression by immunohistochemistry in NF (A and B) and FR50 (C and D) groups in rats with 23 days-old. Arrows indicate basolateral cell membrane.

Fig. 6. (side) MCR kidney expression by immunohistochemistry in NF (A, B, C and D) and FR50 (E, F, G and H) groups in rats with 16 weeks. pp= post proximal segment; p= proximal segment.



For GR expression, the significant difference found in western blot was confirmed and the immunoreactivity is raised in basolateral membrane of all tubular segments, being more significant in post-proximal segments, in 23 days (Figure 7) and 16 week old rats (Figure 8).

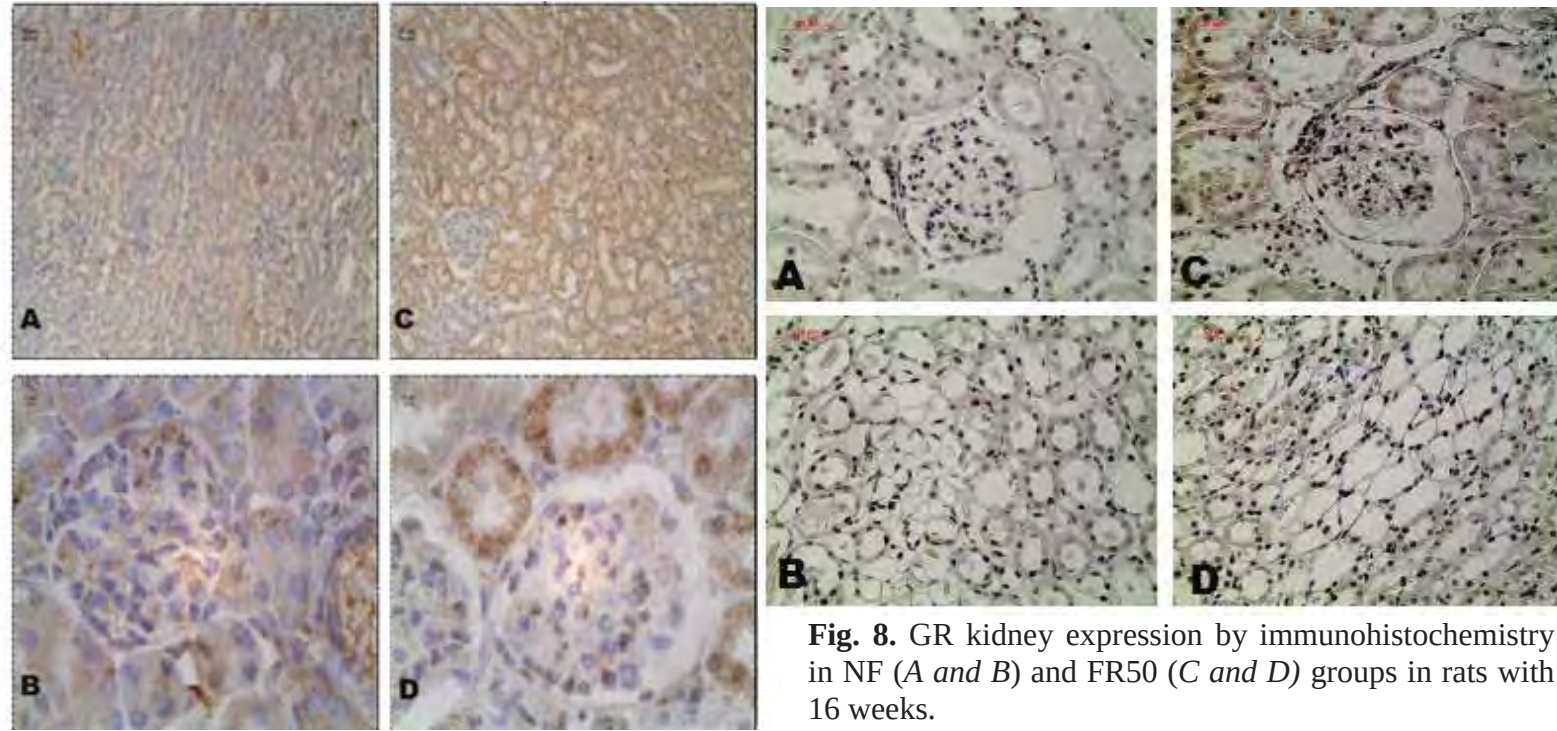


Fig. 8. GR kidney expression by immunohistochemistry in NF (A and B) and FR50 (C and D) groups in rats with 16 weeks.

Fig. 7. GR kidney expression by immunohistochemistry in NF (A and B) and FR50 (C and D) groups in rats with 23 days-old.

Immunostaining showed that AT1 receptors are located in more concentration on the apical surface of proximal tubules, while AT2 receptors are concentrated in the basolateral portion of these tubules. We verified a major reduction in AT1_R expression in 23 days old rat kidneys of FR50 by immunohistochemistry. This reduction occurred in apical surface of tubules, in mesangium and macula dense cells (Figure 9). In accordance with western blot results, the AT2_R reactivity was also more reduced in FR50 group of 23 days old rats (Figure 10). These results are repeated in the offspring of 16 weeks of life.

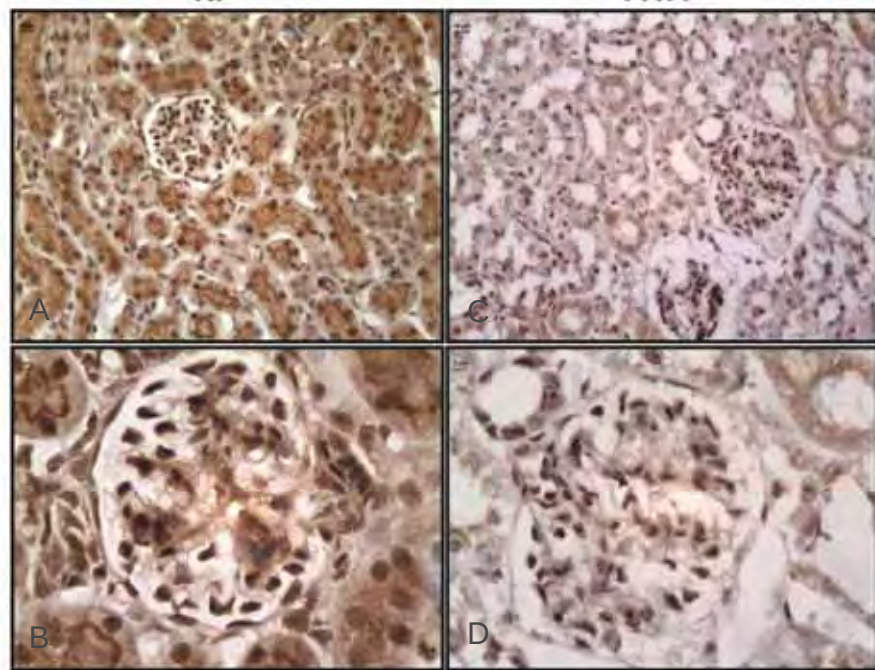


Fig. 9. AT1_R kidney expression by immunohistochemistry in NF (A and B) and FR50 (C and D) groups in rats with 23 days-old.

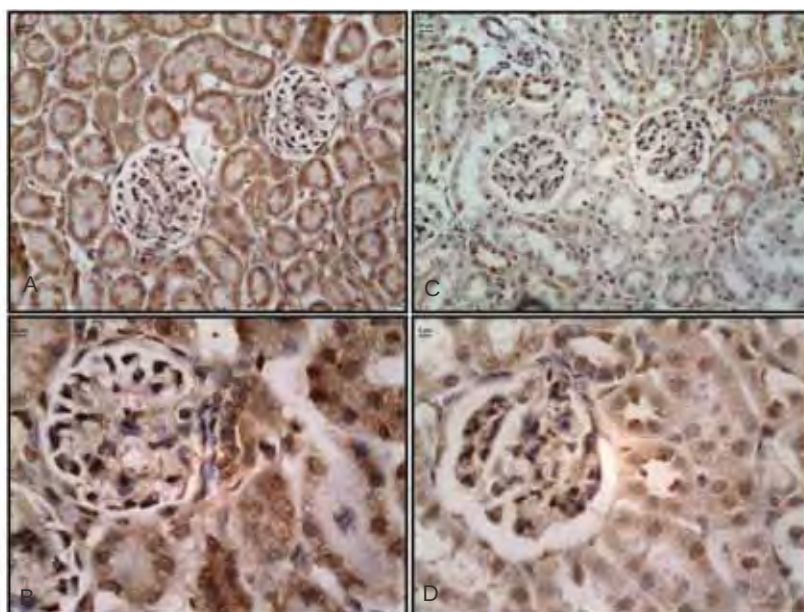


Fig. 10. AT2_R kidney expression by immunohistochemistry in NF (A and B) and FR50 (C and D) groups in rats with 23 days-old.

Discussion

Recent studies have shown that the kidney, during period of development, can be influenced by alterations in the intrauterine environment [21,22]. The data obtained in this study support the hypothesis that malnutrition promotes gestational fetal adaptations that result in changes in the physiology and metabolism of the offspring. The present study confirms that malnutrition during pregnancy programs homogeneous

and consistent appearance of arterial hypertension, since the 6th week of life, in the offspring compared to offspring whose mothers did not suffer such treatment.

The increased expression of glucocorticoid receptors persists throughout adult life, mediating a hypersensitivity to corticosteroids [23,24]. The increased activity of glucocorticoids promote an increase in blood pressure through a series of mechanisms. The most important is the renin-angiotensin system, since glucocorticoids promote increased activity of this system and, thus, cause vasoconstriction and increased peripheral vascular resistance.

In FR50, the reduced expression of angiotensin-II receptors (AT_R) was also observed in the macula densa, suggesting a possible involvement in the tubule-glomerular feedback. Angiotensin II acts in an autocrine and paracrine manner to modulate the proximal tubular transport, and in physiological conditions, promotes proximal sodium reabsorption AT_R-dependent, however, the decrease in tubular AT_R observed in FR50 denotes the impairment of this response with a consequent increase in distal sodium and water distribution caused by the rejection of this ion by the proximal nephron.

In addition, the nephrogenesis requires a delicate balance of several factors that can be changed by the restriction in intrauterine growth [2]. Our group found, among other results, that in rats of 16 weeks of age whose mothers received low-protein diet (6%) during pregnancy, the expression of type 1 and 2 receptors (AT1_R and AT2_R, respectively) of angiotensin II and protein involved in the signaling pathway is reduced [11,25]. In another study, we found that male offspring of mothers submitted to protein restriction during pregnancy, the expression of AT1_R and AT2_R is reduced from 12 days of age, period of finishing nephrogenesis in rats [11,25].

Comparative studies between models of protein restriction (LP) and protein-calorie restriction (FR50) show that the effects of fetal programming *in utero* are more severe in FR50 compared to LP, suggesting that the intensity of nutritional stress is a major factor in the phenotypic expression of these models. FR50 rats presented 17% lower body mass at birth and throughout life, and their blood pressure values increase about 14% compared to control group. In the LP group, the reduction was 8% in birth weight and the increase was 3% in blood pressure (ANDREO *et al.*, unpublished data)

Concluding, we may hypothesize that under conditions of malnutrition during pregnancy, the fetus promotes appropriate modifications to adapt to disruption in the

intrauterine substrate supply. Under normal intake of nutrients after birth, these adaptations may be associated with the development of diseases in adulthood. Excessive fetal exposure to maternal glucocorticoids is responsible for initiating the process of progression to hypertension in adulthood. However, changes in the renin-angiotensin-aldosterone system associated with renal structural and morphological changes may contribute directly to the establishment of hypertension. Additionally, the downregulation of the angiotensin II receptors may result in lack of ability of renal tubules water and salt handling, which in turn may also contribute to hypertension establishment.

Acknowledgments

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