Moderate zinc restriction during fetal and postnatal growth of rats: effects on adult arterial blood pressure and kidney

Anália Lorena Tomat,1 Felipe Inserra,2 Luciana Veiras,1 María Constanza Vallone,1 Ana María Balaszcuk,1 María Angeles Costa,1 and Cristina Arranz1

1 Cátedra de Fisiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Instituto de Química y Metabolismo del Fármaco-Consello de Investigaciones Científicas y Técnicas, Ciudad Autónoma de Buenos Aires; and 2 Instituto de Fisiopatología Cardiovascular, Departamento de Patología, Facultad de Medicina, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina

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In previous works, we have shown that moderate zinc deficiency observed in pregnant women and newborns in developing and developed countries is mainly due to an imbalance between intake and increased requirements and could be a nutritional insult to fetal and postnatal development (28, 20).

Evidence from human and animal studies suggests that the kidney may play a key role in association of maternal undernutrition and intrauterine programming of hypertension (8). Moreover, it is known that renal structure, and specifically nephron number, is a major determinant of blood pressure and renal function (8). Moreover, nephrogenesis in rats begins on embryonic day 12 and is considered completed several days after birth (9, 15, 22).

In previous works, we have shown that moderate zinc deficiency during postweaning growth induced an increase in arterial blood pressure and impaired renal function in adult life. These alterations were associated with decreased activity of the vascular and renal nitric oxide system, an increase in systemic oxidative stress, activation of the renal apoptosis process, and a reduction in the renal filtration surface area (24, 25). Therefore, we hypothesize that zinc deficiency during fetal life and early postnatal growth could be an adverse environment for the development of the cardiovascular and renal systems. Moreover, prenatal effects induced by zinc deficiency could not be totally reversed by an adequate zinc intake during growth and could predispose to diseases in adult life. Therefore, the purpose of the present study was to evaluate the effects of moderate zinc deficiency during fetal life, lactation, and/or postweaning growth on systolic blood pressure (SBP), renal function and morphology, and renal oxidative stress state in adult life.

MATERIALS AND METHODS

Animals and study design. Female Wistar rats from the breeding laboratories of the Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Argentina) were mated by exposure to Wistar males during 1 wk. Immediately afterwards, female rats were randomly fed either a moderately zinc-deficient diet (L; 8 ppm) or a control zinc diet (C; 30 ppm) during the pregnancy and lactation periods. After birth, no more than nine rat pups remained with each mother. After weaning, male offspring of each group of mothers were fed low or control zinc diet. Systolic blood pressure, creatinine and metabolic alterations in adult life (1). Zinc is an essential trace element required by all living organisms for many physiological functions, including growth, development, and reproduction (28). Maintenance of discrete subcellular pools of zinc is critical for the functional and structural integrity of cells and is involved in maintenance of the physiological barrier function, reduction of oxidative stress, and inhibition of apoptosis (27, 33). Moderate and marginal zinc deficiency observed in pregnant women and newborns in developing and developed countries is mainly due to an imbalance between intake and increased requirements and could be a nutritional insult to fetal and postnatal development (28, 20).

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necessary nutrients, except zinc content, to meet requirements for the periods of rat pregnancy, lactation, and growth according to AIN-93 recommendations as described previously (24, 25).

Animal handling and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the American Physiological Society “Guiding Principles in the Care and Use of Animals,” and with the 6344/96 regulation of Argentinean National Drug Food and Medical Technology Administration (ANMAT). Animals were allowed food and deionized water ad libitum. All the laboratory material was previously washed with nitric acid (20%) and deionized water. Male offspring were housed separately in plastic cages in a humidity- and temperature-controlled environment, illuminated with a 12:12-h light-dark cycle.

SBP was measured indirectly in awake animals by the tail cuff method using a Grass polygraph at 60 days after weaning, as described previously (24, 25). At day 60 after weaning, blood samples were collected from the rats’ tails and animals were placed in plastic metabolic cages to collect 24-h urine and feces samples. Urine volume was determined gravimetrically. Plasma and urinary creatinine levels were measured by a colorimetric method (Wiener Laboratory). Creatinine clearance was calculated to estimate the glomerular filtration rate (GFR). Protein concentration in urine was assayed by the method of Lowry et al. (10). Concentration of zinc in plasma and blood samples obtained on day 80 after weaning were measured by atomic absorption spectrophotometry. The following was used: zinc calibration standards, air acetylene flame, 0.5-nm slit, wavelength of 213.9 nm, as described previously (24, 25). At the end of the dietary treatment, rats were killed by cervical decapitation, and both kidneys were immediately removed and weighed. Morphometric studies, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays, and immunohistochemical analyses were performed in the right kidney. Meanwhile, the left kidney was used to determine zinc concentration (24) and to measure antioxidant and oxidant systems.

Histological evaluation, immunolabeling, and TUNEL assay. Descapsulated right kidneys were cut longitudinally, fixated in phosphate-buffered 10% formaldehyde, pH 7.2, and embedded in paraffin wax. The following was used: polyclonal mouse anti-SMA (Sigma), anti-mouse α-SMA (Sigma); immunolabeling was revealed with a modified avidin-biotin-peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories); nitric acid proanalysis (Merck); plasmatic and urinary creatinine levels by colorimetric method (Wiener Laboratory); DeadEnd Colorimetric TUNEL System, a nonradioactive kit designed to end-label the fragmented DNA of apoptotic cells, was used as previously described (24). The number of TUNEL-positive cells per cortical area was counted in 20 visual fields (magnification 400) for each rat.

Measurement of antioxidant and oxidant stress systems in renal tissue. Lipid oxidative damage was assessed measuring the extent of formation of 2-thiobarbituric acid reactive substances (TBARS; nmol/mg protein) (3). Glutathione content was measured and was expressed as milligram per milligram protein (23). SOD was assayed by measuring the ability of the homogenate to inhibit autoxidation of epinephrine and was expressed as units of SOD per milligram protein (12). Catalase activity was determined by the conversion of hydrogen peroxide to oxygen and water and was expressed as picoeuler per milligram protein (11). The assay described by Flohé and Gunzler (6) was used to measure the glutathione peroxidase activity (GPx) and was expressed as nanomoles per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al. (10).

Statistical analysis. All values are expressed as means ± SE. Prism (Graph Pad Software) was used for statistical analysis. Data were analyzed using one-way ANOVA followed by Bonferroni multipletest post hoc test. Linear regression analysis was used to determine the relationship between SBP and GFR, glomerular number, and glomerular filtration surface areas. P < 0.05 was considered a significant difference.

Chemicals, reagents and equipment. The following was used: α-SMA antibodies, periodic acid-Schiff reagent, and Masson’s trichrome. Renal morphometric parameters were determined in 10 consecutive cortical and juxtamedullary areas from two renal sections that were not consecutive and belonged to different parts of the kidney. The number of glomeruli was measured at ×100 magnification and the area of each field was 1.453 mm². The glomerular areas and the number of nuclei per glomerulus were determined at ×400 magnification, each field corresponding to an area of 1.367 mm². The filtration surface area was calculated as the product of mean glomerular capillary area by the number of glomeruli per area.

Kidney sections were subjected to α-smooth muscle actin (α-SMA) immunohistochemistry and to collagen-specific stain PicroSirius Red to determine the presence of fibrosis in the renal cortex, as previously described (24). Cortical areas were analyzed at ×400 magnification. Expression of α-SMA in the renal cortex was scored as follows: 0 = normal staining confined only to smooth muscle cells of blood vessels; 1 (mild) = additional weak staining in the peritubular interstitium, glomeruli, and periglomerular structures; 2 (moderate) = moderate segmental or focal staining of peritubular interstitium, periglomerular structures, and a small minority of glomeruli; 3 (severe) = strong staining, <25% of the cortical area, in the majority of glomerular cells and tubular and peritubular structures; and 4 (very severe) = strong staining, >25% of the cortical area, in the majority of glomerular cells and tubular and peritubular structures.

PicroSirius Red staining in the renal cortex was scored as follows: 0 = normal and slight staining surrounding tubular, glomerular, and vascular structures; 1 (mild) = weak staining, that doubles normal label, surrounding tubular, glomerular, and vascular structures; 2 (moderate) = moderate staining in the peritubular interstitium and inside glomeruli; 3 (severe) = strong staining that replaces glomerular and tubular structures, compromising <25% of the cortical area; and 4 (very severe) = strong staining that replaces glomerular and tubular structures, compromising >25% of the cortical area. A score was assigned to each section, mainly reflecting the changes in the extent rather than the intensity of staining.

The DeadEnd Colorimetric TUNEL System, a nonradioactive kit designed to end-label the fragmented DNA of apoptotic cells, was used as previously described (24). The number of TUNEL-positive cells per cortical area was counted in 20 visual fields (magnification ×400) for each rat.

Histological studies and TUNEL assays were analyzed using a Nikon E400 light microscope equipped with a digital camera connected to the Image-Pro Plus 4.5.1.29 software. The measurements were performed blindly and under similar light, gain, offset, and magnification conditions.

RESULTS

At the moment of weaning, male offspring of L mothers exhibited a significant difference in body weight compared with male offspring of C mothers (C: 50.2 ± 0.9 vs. L: 46.8 ± 0.9 g, P < 0.01). However, at the end of the dietary treatment (60 days) there were no significant differences among the dietary groups (C: 400 ± 8; Cl: 386 ± 7; Lc: 373 ± 11; Ll: 379 ± 10 g, ns). Furthermore, because daily food intake in all groups was similar (C: 22.17 ± 0.96; Cl: 21.34 ± 0.79; Lc: 22.88 ± 1.06; Ll: 23.01 ± 1.01 g/day, not significant), it was not necessary to pair feed control rats. The mothers fed either
a low or a control zinc diet exhibited similar daily food intake during the experimental period (L: 32.1 ± 5.7 vs. C: 26.7 ± 3.21 g/day, not significant).

LI and Cl animals showed lower zinc concentrations in the kidneys (Cc: 27 ± 1; Cl: 19 ± 1; Lc: 25 ± 2; Li: 18 ± 1 μg/g tissue), plasma (Cc: 168 ± 13; Cl: 110 ± 7; Lc: 140 ± 10; Li: 98 ± 8 μg/dl), and feces (Cc: 756 ± 155; Cl: 100 ± 14; Lc: 702 ± 138; Li: 150 ± 25 μg/day) than Cc and Lc rats at 60 days after weaning (*P < 0.05 vs. Cc, †P < 0.001 vs. Lc).

Animals exposed to a moderately zinc-deficient diet during preweaning and/or postweaning growth (LI, LC, and Cl groups) exhibited higher values of SBP and lower levels of GFR at the end of the dietary treatment compared with the Cc group (Fig. 1). There were no significant differences in SBP and GFR levels among Cc and Cl groups. Moreover, there was a positive correlation between SBP and the number of cortical (*P < 0.05 vs. Cc, †P < 0.001 vs. Lc).

Kidney weight and morphometric parameters are shown in Table 1. There were no differences in kidney weight among the experimental groups. The cortical and juxtamedullary zone of LI, LC, and Cl kidneys showed less number of glomeruli per area compared with Cc rats. However, these morphometric parameters were higher among LI, LC, and Cl groups. Moreover, the total glomerular area, the glomerular capillary area, and the filtration surface areas were reduced in cortical and juxtamedullary zones of the rats exposed to moderate zinc deficiency during preweaning and/or postweaning growth. However, these morphometric parameters were higher in LC animals compared with LI and Cl groups. LI rats presented, in cortical zone, a smaller glomerular capillary area than Cl group. The number of nuclei per glomeruli was lower in Cl and LI rats than in Cc and Lc ones. A positive relationship was observed between GFR and the number of cortical (*r = 0.882, P < 0.001) and yuxtamedullar (*r = 0.811, P < 0.001) glomeruli. Moreover, there was a positive correlation between GFR and the cortical (*r = 0.865, P < 0.001) and yuxtamedullar (*r = 0.820, P < 0.001) renal filtration surface areas.

SBP was negatively correlated with the number of cortical (*r = 0.867, P < 0.001) and yuxtamedullar glomeruli (*r = 0.834, P < 0.001) at the end of the experimental protocol. There was also a negative correlation between SBP and the cortical (*r = 0.707, P < 0.01) and yuxtamedullar (*r = 0.854, P < 0.01) renal filtration surface areas.

Areas of positive α-SMA were confined only to smooth muscle cells of blood vessels in Cc and Cl renal cortex at the end of the experimental protocol. By contrast, Sirius Red and α-SMA staining showed signs of fibrosis in glomeruli, tubules, and peritubular interstitium of LI and LC kidneys (Sirius Red staining score: Cc = 0.5 ± 0.1; Cl = 0.7 ± 0.2; Lc = 2.0 ± 0.1; LI = 2.1 ± 0.1; α-SMA staining score: Cc = 0.25 ± 0.1; Cl = 0.5 ± 0.1; Lc = 1.9 ± 0.1; LI = 2.1 ± 0.1), (Fig. 2, A and B).

Urinary protein excretion, determined at the end of the dietary treatment, was higher in LI and LC animals compared with the Cc and Cl diet groups. However, LI animals showed higher urinary protein concentrations, compared with the other groups (Cc: 3.09 ± 0.46; Cl: 2.73 ± 0.54; Lc: 5.26 ± 0.52%‡; LI: 14.5 ± 2.09%‡ mg/day. 100g. *P < 0.001 vs. Cc; †P < 0.01 vs. Lc, ‡P < 0.01 vs. Cl).

Examination of TUNEL-stained renal sections at the end of the experimental period revealed an increased number of apoptotic cells in cortical areas of rats exposed to a moderately zinc-deficient diet, during any period of life, compared with the Cc group (Fig. 2C). Moreover, LI rats exhibited a higher number of apoptotic cell in tubules and glomeruli of the renal cortex than the Cl and Lc groups (Cc: 5.2 ± 0.5; Cl: 20 ± 5%; Lc: 14 ± 4%; LI: 50 ± 4%‡ cells/area*P < 0.01 vs. Cc, †P < 0.01 vs. Lc, ‡P < 0.01 vs. Cl).

Glutathione concentration, TBARS levels, SOD, catalase, and GPx activity measured in the renal tissue at the end of the experimental period are shown in Table 2. Animals exposed to moderate zinc deficiency (LI, LC, and Cl) exhibited higher TBARS levels compared with the Cc group. Moreover, LI animals showed higher TBARS concentration than the Cl and Lc groups. Meanwhile, LI and Cl renal tissues showed lower levels of glutathione concentration, catalase activity, and GPx activity compared with the Cc and Lc groups. However, GPx activity was still diminished in the renal tissue of Lc rats compared with the Cc ones. Nevertheless, there were no significant differences in SOD activity among the groups.

DISCUSSION

The present data show that moderate dietary zinc restrictions in mothers during pregnancy and lactation and/or in offspring’s during postweaning growth induce functional and morphological adaptations that result in long-term cardiovascular and renal effects in adult life.

Previously, many reports have shown that severe zinc deficiency reduces food intake and causes growth retardation in rats (28, 14). We observed that animals exposed to moderate zinc deficiency during fetal life and the lactation period showed lower body weight at the moment of weaning, which was not
secondary to reductions in the mother’s food intake. During the first stage of the lactation period, zinc deficiency in offspring would be maintained by the mother’s milk. Even though we have not determined milk composition, L mothers showed low zinc plasma concentration at weaning (data not shown). This is a good marker of milk zinc deficiency given that the mammary gland is the physiological link between circulating and milk micronutrients (13). Moreover, on the basis of previous reports that showed that zinc deficiency reduces milk zinc levels but does not affect milk fat and total protein concentration, we think that the quality of milk, except zinc content, would be adequate (7, 34).

Even though we could not determine the precise level of zinc deficiency in fetal life induced by the low zinc diet in the L mothers, both Lc and Li offspring showed an increased blood pressure in adult life. Our data support many epidemiological studies proposing that factors present in the prenatal environment (1).

Moreover, underlying renal mechanisms may be responsible for the increase in arterial blood pressure induced in response to moderate zinc deprivation during fetal life, lactation, and/or postweaning growth. This hypothesis is supported by our data that shows a decrease in GFR in the deficient groups and a close negative correlation between GFR and SBP. These results are in agreement with our previous studies that showed that rats exposed to moderate zinc deficiency during postweaning growth presented an increase in SBP related to a decrease in GFR, impairment in the renal and vascular nitric oxide system, and alterations in renal morphology (24, 25).

In the present study, we have also shown that renal dysfunction is associated with a reduction in nephron number and glomerular areas, independently of the moment of growth that animals were exposed to the deficiency. In rats, nephrogenesis occurs during the last third of gestation and continues for several days after birth, as it has been reported by numerous authors (9, 15, 22).

Adaptations to zinc restrictions that occur during critical periods of renal development may result in a reduction in renal filtration surface area and a higher risk of alterations in arterial blood pressure regulation. This hypothesis is supported by the positive relationship between glomerular number and GFR, and the negative correlation between the number of glomeruli and SBP observed at the end of the dietary treatment. The link between a marked reduction in nephron number and a significant elevation in blood pressure has also been demonstrated in many animal models that induce an adverse fetal environment (1).

Zinc deficiency induced similar morphometric changes in the most mature nephrons in a juxtamedullary position and in the less mature nephrons in the superficial region of the renal cortex. An adequate zinc diet during postweaning growth mitigated the alterations in glomerular areas and the number of glomerular nuclei induced by zinc deficiency during fetal life and lactation. However, these improvements were not enough to normalize GFR and/or SBP levels.

Moreover, many reports show that the glomerular overall tuft volume, the capillary length, the capillary surface area, and GFR increase rapidly and continuously until at least day 60 (15, 30). So we think that an adequate zinc intake is necessary to induce a satisfactory renal growth process. Besides these renal changes, moderate zinc deficiency during the fetal and lactation periods of life induced early signs of fibrosis in different structures of the renal cortex that were not overcome with a control diet during postweaning growth.

Moreover, the animals exposed to zinc deficiency during the entire experimental period showed higher excretion of urinary proteins than the other groups, probably indicating a dysfunction in the glomerular filtration barrier. Furthermore, the alter-
ations induced by zinc deficiency on the glomerular filtration barrier during fetal life and lactation could partially be restored by a control diet during postweaning life, demonstrating the importance of this mineral for adequate renal development during early stages of growth.

Apoptosis is an active form of cell death that plays an important role in tissue maintenance and organ homeostasis (32). Previous reports showed that the increased appearance of programmed cell death would depend on the changes in labile cellular zinc pools, which are influenced by zinc deprivation.

Table 2. Antioxidant and oxidant stress systems

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<th>Cc</th>
<th>Cl</th>
<th>Lc</th>
<th>Ll</th>
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<tr>
<td>GLUT, mg/mg protein</td>
<td>2.1±0.2</td>
<td>1.3±0.02†</td>
<td>1.9±0.05</td>
<td>1.4±0.07†</td>
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<tr>
<td>TBARS, nmol/mg protein</td>
<td>0.16±0.02</td>
<td>0.46±0.02†</td>
<td>0.38±0.05*</td>
<td>0.66±0.08‡</td>
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<tr>
<td>SOD activity, U/mg protein</td>
<td>10.3±1.7</td>
<td>11.2±1.5</td>
<td>10.7±1.5</td>
<td>12.1±1.1</td>
</tr>
<tr>
<td>CAT activity, pmol/mg protein</td>
<td>2.1±0.4</td>
<td>1.2±0.1†</td>
<td>2.6±0.1</td>
<td>1.4±0.4†</td>
</tr>
<tr>
<td>GPx activity, pmol/min/mg protein</td>
<td>8.6±1.8</td>
<td>3.1±0.9†</td>
<td>6.4±0.5*</td>
<td>3.5±0.3†</td>
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Values are means ± SE; n = 10 per group. GLUT, glutathione concentration; TBARS, 2-thiobarbituric acid reactive substances concentration; SOD, superoxide dismutase activity; CAT, catalase activity; GPx, glutathione peroxidase activity; *P < 0.05 vs. Cc; †P < 0.05 vs. Cl, ‡P < 0.05 vs. Lc. Data were analyzed at the end of the experimental period using one-way ANOVA followed by a Bonferroni post hoc test.
(26, 27, 28). The present study showed an increase in the number of apoptotic cells in cortical tubules and glomeruli of rats exposed to moderate zinc deficiency. Therefore, we propose that a reduction in renal zinc content could induce modifications in labile zinc pools of renal cortical cells, increasing the risk of apoptosis and leading to a decrease in the number and size of nephron units, and consequently in the GFR. Moreover, animals exposed to zinc deficiency during fetal life and lactation but fed an adequate zinc diet during postweaning growth, showed similar renal zinc content as control rats, and this partially reversed the effects on renal apoptosis and glomerular filtration areas. These effects would probably be completely reversed if these animals were fed a control diet during a longer period of postweaning life or a supplemented zinc diet during the same experimental period. Moreover, several local factors such as the renin angiotensin system, prostaglandins, growth hormone, and insulin-like growth factors play an important role in this process of nephron maturation (4, 31). Therefore, we cannot discard the prospect that some of these factors could be altered in our experimental model.

One of the mechanisms that may underlie the antiapoptotic effect of zinc is the decrease in cellular oxidative stress. Previous studies have demonstrated that zinc can act as an antioxidant by binding to membrane sites that might otherwise bind redox-active metals (such as copper and iron), protecting sulphhydryl groups from oxidant and by being an essential component of the SOD enzyme and of the metallothioneins. It has been reported that zinc may also protect cells from oxidative damage via its effects on glutathione, the main intracellular anti-oxidant (18, 19, 26, 33).

Our study demonstrated that moderate zinc deficiency during pre- and/or postweaning growth induced renal oxidative damage, as indicated by the increase of lipid peroxidation, the reduction in glutathione levels, and the decrease in GPx and catalase activities in the kidney. However, we found that moderate zinc deficiency did not modify renal SOD activity. The effects of zinc deficiency on SOD activity are controversial. Meanwhile, several studies have shown that zinc deficiency leads to a significant decrease in SOD activity (16, 21), others reported an increase (17, 29) or no changes (2, 5) in the activity of this enzyme in different tissues. Our results are not surprising considering that zinc is a constituent of SOD and that the enzyme active site is not stringent to zinc and it may possibly be replaced by other metals (17).

Oxidative damage depends on the rate of free radical production and on the level of protection exerted by the antioxidant defense system (29). Zinc deficiency induced a decrease in catalase and GPx activities, probably generating an imbalance between the level of SOD end products and the capacity of the renal tissue to metabolize hydrogen peroxide. Therefore, we propose that these effects may have contributed to the reduction in glutathione levels and to the increase in renal lipid peroxidation end products in the kidney. Moreover, the increase in TBARS and reactive oxygen species would trigger renal cell apoptosis and could also play a role in the renal morphological damage associated with zinc deficiency.

Restoring renal zinc content through an adequate zinc diet during postweaning growth was successful in normalizing catalase activity and glutathione content, but it could not return either GPx activity or TBARS concentration completely to normal. Therefore, we suggest that the increase in renal zinc content observed in this group could induce a decrease in reactive oxygen species production that leads to a reduction in the number of renal apoptotic cells and an increase in glomerular filtration areas compared with those animals exposed to zinc deficiency during all the experimental period.

In conclusion, based on the results of the present study and our previous results (24), zinc deficiency during any period of growth could induce an increase in arterial blood pressure associated with alterations in renal function and morphology. However, moderate zinc deficiency all along growth, induced greater renal damage accompanied by higher apoptotic and lipid peroxidation process than when it was induced only during preweaning or postweaning life.

Perspectives and Significance

Micronutrient undernutrition during critical periods of growth has become an important health issue in developing and developed countries, particularly in pregnant women and children having an imbalanced diet. This study highlights the impact of moderate zinc restrictions during fetal and postnatal life on the renal and cardiovascular systems in adult life. The molecular, hormonal and humoral mechanisms through which this deficiency during prenatal and postnatal growth exerts its effects on different organs of female and male offspring, including the kidney, remain to be further elucidated.

Nevertheless, the fact that even seemingly minor influences such as composition of diet during pregnancy, lactation, and postweaning growth can have major consequences in adult life underscores the critical importance of perinatal care optimization for a better management and prevention of adult cardiovascular and renal diseases. Awareness of healthy eating, exercise, and minimization of cardiovascular risk factors, therefore, needs to begin in infancy. Since the risk of many common diseases in adulthood can be determined during growing periods of life, it would be important to test whether adequate or supplemented diets in adult life may or may not reverse the physiological changes.

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