

# EFFECTS OF NUTRITIONAL COPPER DEFICIENCY ON ADULT NON-PREGNANT AND PREGNANT RATS AND THEIR NEWBORNS

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## SUMMARY

The effects of nutritional copper deficiency on weight gain, food intake and fertility of pregnant and non-pregnant adult Wistar rats, and weight of newborns, were studied. The adult animals were subjected to diets with different copper content (control-5 ppm; moderate deficiency -1.2 ppm; severe deficiency -0.3 ppm) for six weeks (non-pregnant) and nine weeks (pregnant). No effect on weight gain or appetite was seen in the groups of animals subjected to copper deficiency. Fertility, as evaluated by the number of conceptuses and liverborns of the pregnant animals, and weight of newborns also, were not impaired.

These data show that not only the extent of deficiency, but also the period during which it is imposed, as well as its duration, condition the appearance of alterations of this deficiency.

## INTRODUCTION

Biological studies on copper date from 1818, when Bucholz discovered that it was one of the vegetal and animal tissue components (1). Work on its essentiality, however, started only in 1990 with the observation of non-responsive to iron anemia in animals receiving a milk diet (2).

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Copper was recognized as an important component of human and animal diets in the second decade of this century, when the works of Hart *et al.* (3) and Waddell *et al.* (4) confirmed Abderhalden's observations in 1900 (2). These authors demonstrated that weanling female rats fed exclusively a milk diet became anemic, non-responsive to therapeutic iron administration, unless they received added copper.

Although there were speculations about a possible copper deficiency in humans, only in the 1960s was its clinical importance demonstrated in studies on marasmic Peruvian infants, described by Cordano, Baertl and Graham (5).

Organic copper function is connected to its role as a cofactor and also as a component of multiple enzymatic systems (6).

Experimental studies with different animals subjected to nutritional copper deficiency in adulthood and during pregnancy, have shown divergent results (7, 8). High incidence of fetal resorption and stillborns (9, 10), as well as congenital abnormality and post-natal mortality (11) have been described. Masters *et al.* (8)—studying copper deficiency in rats during pregnancy— did not observe differences regarding number of newborns and weight of mothers and newborns, when compared to a control group.

Due to the importance of this ion for growth and development of the organism, we proposed to study the effects caused by diets containing different copper concentrations on adult non-pregnant and pregnant rats and their newborns.

## MATERIAL AND METHODS

### 1. *Animals*

Female 90-day old Wistar rats, weighing between 180 and 220 g, were used. These animals after remaining in collective cages and receiving a copper adequate diet for seven days, were placed in individual cages and divided at random to be fed one of the following three types of diets during six weeks:

Diet (S) - Severe copper deficiency = 0.3 ppm.

Diet (M) - Moderate copper deficiency = 1.2 ppm.

Diet (C) - No copper deficiency (control) = 5.0 ppm.

The control diet composition was based on the Fox and Briggs (12) and Lajolo, Franca and Zucas (13) models. The protein content was 21%, and copper, 5.0 parts per million (ppm).

The deficient diet composition was basically the same as that of the control, differing only in its copper content.

The animals received diets *ad libitum*, with deionized water, in drinking bottles with glass tips.

#### 1.1 *Groups*

As of the 97th day, different groups of animals were established, as shown in Figure 1. Animals of the non-pregnant groups (NP), named according to the diet received (severe (NPS), moderate (NPM) or control (NPC)), were

sacrificed after completing six weeks of study. The pregnant groups (P) were constituted by animals that, after said period (6 weeks), were caged with adult animals daily, for 12 hours during the night, without food, receiving only deionized water *ad libitum*. After evidence of mating (presence of spermatozoa in vaginal smears), the animals were divided into seven groups according to the different diet combinations (Figure 1) and sacrificed on the 21st day of pregnancy.

Newborn animals (NB) were obtained by caesarian section on the 21st day or pregnancy, constituting different groups according to those of their mothers (Figure 1).

### 1.2 *Animal observation*

From the 97th day on, there were daily cage changes and the animals were observed for four variables: 1) food intake; 2) alterations of behavior; 3) alterations of hair, ulcers on tail and feet, and 4) mortality. Animals were weighed ever five days. Pregnant animals were weighed on the day of mating evidence (day zero) and on the 4th, 7th, 11th, 14th, 18th and 21st day of pregnancy. The unconsumed food for each animal was weighed and replaced by a new known amount (30g daily), and the difference recorded to obtain daily intake for each animal.

As the treatment for non-pregnant rats and those that became pregnant was the same during the first six weeks, these animals were considered to be a single group concerning weight and amount of diet intake, differing only as to the kind of diet received.

### 2. *Cages*

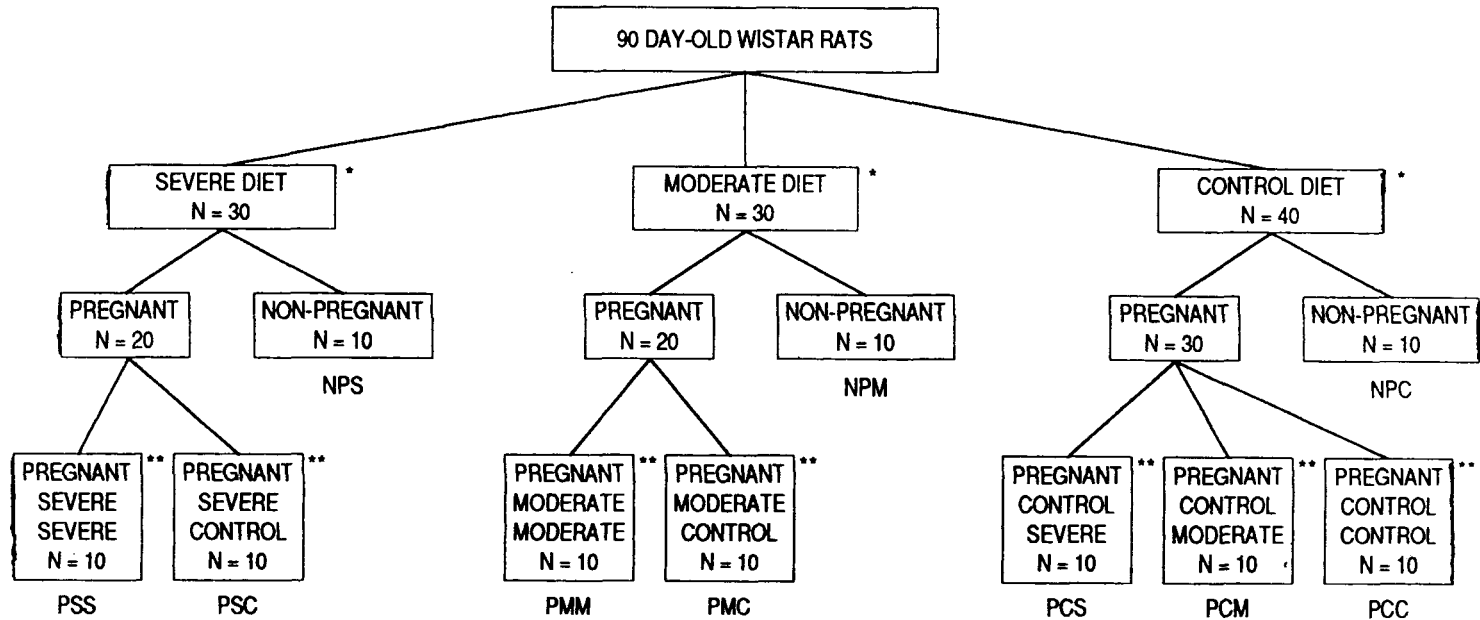
The cages used at the beginning of the experiment up to the 97th day of life, were made of polypropylene and allowed the housing of up to six animals with space for moving around. From the 97th day until the end of the experiment, the rats were lodged in individual polypropylene cages with glass bottoms and steel covers to avoid contamination with environmental copper. The containers for food and water, both made of glass, were fastened to the cover.

### 3. *Statistical Methods*

The following statistical analyses were applied:

- Variance analysis for independent groups (14). When there was a significant difference, it was complemented by Scheffe's test (14).
- Variance analysis for non-independent groups (14). In the presence of a significant difference, this analysis was complemented by the Tukey's test (14).
- Chi-square partition test (15).
- Variance analysis by Kruskal-Wallis test (16).

Significance level of results was considered to be  $p \leq 0.05$ .



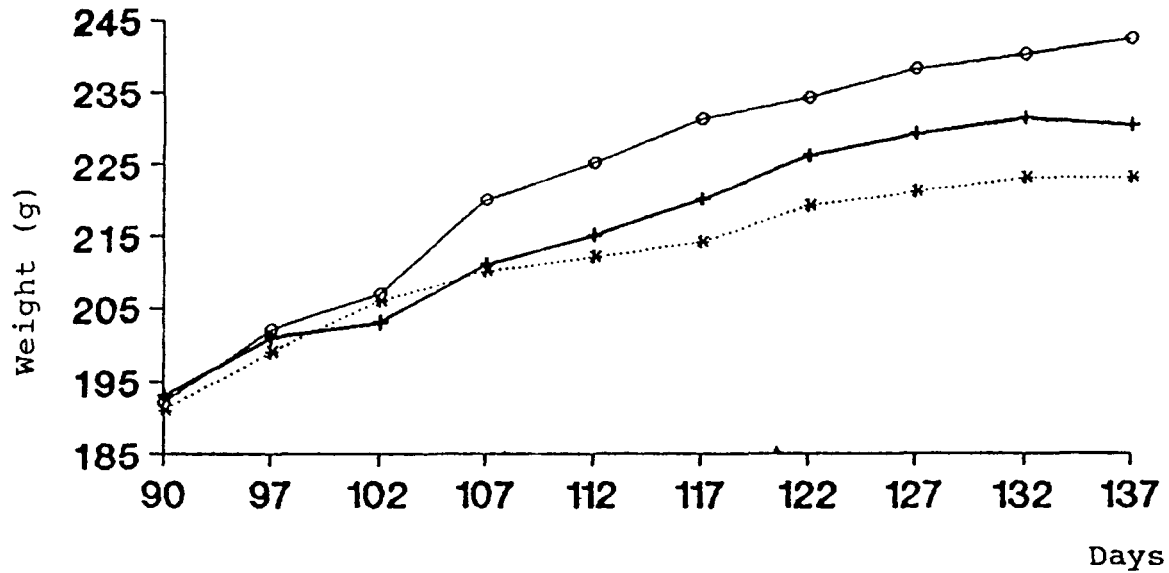
\* Animals of these groups received the diet for 6 weeks.

\*\* Animals of these groups received the diet for additional 3 weeks.

**FIGURE 1**

**Distribution of non-pregnant (NP), pregnant (P) and newborn (NB) animals in the different groups according to the copper deficient diet received: (S - severe; M - moderate; C - control).**

**For details see text**



**FIGURE 2**

**Weight gain of animals subjected to severe copper deficiency (...\*) moderate copper deficiency (—°—) and control (— + —) during the first six weeks of the study. Significant difference ( $p < 0.05$ ) was observed for the 107th up to the 137th day, Scheffe's test showing  $S < M$**

## RESULTS

The weight gain of the animals during the first six weeks is presented in Figure 2. We observed weight gain from the 90th day on, which was significant up to the 10th day for female rats fed the S diet, and up to the 112th day for those fed M and C diets. Weight gain of rats receiving M diet was significantly greater than that of those receiving S diet from the 107th day on.

Weekly mean food intakes during the first six weeks are shown in Table 1. Food consumption decreased with time mainly from the 3rd week on. Food intake by the animals that were fed the C diet was greater than that of those receiving S diet during the 1st, 3rd, and 5th weeks.

TABLE 1

MEAN WEEKLY DIET INTAKE IN GRAMS, ACCORDING TO THE GROUP STUDIED-MEAN  $\bar{x} \pm$  STANDARD ERROR (SE)

Diet	S	M	C
	(N = 30)	(N = 30)	(N = 40)
Weeks	$\bar{x} \pm$ SE	$\bar{x} \pm$ SE	$\bar{x} \pm$ SE
1	12.76 $\pm$ 1.80	13.97 $\pm$ 1.62	14.13 $\pm$ 2.55
2	12.25 $\pm$ 1.45	12.30 $\pm$ 1.37	13.08 $\pm$ 2.33
3	11.17 $\pm$ 1.57	11.51 $\pm$ 1.37	12.53 $\pm$ 1.86
4	11.16 $\pm$ 1.46	11.63 $\pm$ 1.57	11.98 $\pm$ 1.73
5	10.84 $\pm$ 1.36	11.39 $\pm$ 1.70	12.10 $\pm$ 1.42
6	10.79 $\pm$ 1.35	10.78 $\pm$ 1.42	11.16 $\pm$ 1.42

Analysis of variance for non-independent groups.

S = 1 > 3, 4, 5, 6; 2 > 5, 6 ( $p > 0.01$ ).

M = 1 > 2, 3, 4, 5, 6; 2 > 6 ( $p > 0.01$ ).

C = 1 > 3, 4, 5, 6; 2 > 6 ( $p > 0.01$ ).

Analysis of variance for independent groups.

1st, 3rd, 5th weeks: C > S ( $p > 0.05$ ).

Non significant difference was found between the other groups.

During pregnancy, there was a significant weight gain for all animal groups until the 21st day of pregnancy (Table 2). Rats of group PMC presented a higher weight gain than rats of group PSS at days 0, 4, 7, 14 and 21.

Weight gain percentage, taking as reference the initial weight (day zero) of pregnant rats, was not significant between the groups, in spite of being numerically greater in group PCC (data not shown).

Mean daily food intake, evaluated weekly during pregnancy (Table 3) was significantly greater during the 2nd and 3rd weeks as compared to the 1st, for group PSS, and during the 2nd week as compared to the 1st, for animals of group PCC. Comparison between the groups showed that during the 3rd week

**TABLE 2**

**PONDERAL EVOLUTION IN GRAMS-MEAN ( $\bar{x}$ ) AND STANDARD DEVIATION (SD) OF ANIMALS OF GROUPS. PSS, PSC, PMM, PMC, PCS, PCM, PCC**

Days of pregnancy	Groups	PSS	PSC	PMM	PMC	PCS	PCM	PCC
		(N = 10)	(N = 10)	(N = 10)	(N = 10)	(N = 10)	(N = 10)	(N = 10)
		$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
0		209.7 ± 17.3	217.5 ± 19.6	224.6 ± 11.0	233.1 ± 17.4	231.2 ± 19.0	225.9 ± 15.9	214.8 ± 9.4
4		223.0 ± 16.7	230.6 ± 19.6	238.5 ± 9.2	253.1 ± 24.1	244.5 ± 18.3	238.5 ± 16.5	231.1 ± 6.7
7		230.1 ± 19.9	238.0 ± 21.6	244.7 ± 9.2	259.9 ± 24.4	251.9 ± 19.9	245.2 ± 18.0	239.1 ± 9.9
11		240.5 ± 19.5	248.3 ± 22.7	258.7 ± 14.4	269.2 ± 28.1	264.6 ± 21.1	255.3 ± 21.8	253.3 ± 10.4
14		249.2 ± 16.9	260.7 ± 24.6	267.7 ± 11.8	277.4 ± 26.9	276.6 ± 20.5	266.8 ± 22.9	268.5 ± 9.3
18		272.7 ± 18.5	287.6 ± 23.0	295.2 ± 13.0	293.8 ± 35.1	301.7 ± 21.7	295.2 ± 22.2	288.6 ± 9.9
21		303.5 ± 26.7	319.2 ± 23.8	323.9 ± 17.9	348.9 ± 33.4	332.4 ± 22.7	325.7 ± 25.8	310.8 ± 14.7

Analysis of variance for non-independent groups showed significant differences for all groups ( $p < 0.05$ ) and Tukey's test showed:

PSS, PSC, PMM, PCS, PCM, PCC

0 < 11, 14, 18, 21

4 < 18, 21

7 < 18, 21

11 < 18, 21

14 < 21

18 < 21

PMC

0 < 14, 18, 21

4 < 18, 21

7 < 21

11 < 21

14 < 21

18 < 21

Analysis of variance for independent groups showed significant differences ( $p < 0.05$ ) for days 0, 4, 7, 14, 21 and Scheffe's test showed PMC > PSS.

For details see text.

TABLE 3

MEAN WEEKLY INTAKE OF DIETS IN GRAMS-MEAN ( $\bar{x}$ ) AND STANDARD ERROR (SD) ACCORDING TO THE GROUP STUDIED DURING THE GESTATIONAL PERIOD

Week of pregnancy	Groups	PSS	PSC	PMM	PMC	PCS	PCM	PCC
		$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$
1		12.68 ± 1.35	12.40 ± 1.18	12.63 ± 2.46	12.93 ± 1.24	13.09 ± 1.11	12.67 ± 1.85	12.87 ± 1.49
2		12.88 ± 1.17	14.32 ± 1.42	14.52 ± 1.64	13.92 ± 1.75	13.79 ± 0.96	14.23 ± 1.31	14.66 ± 1.33
3		13.43 ± 1.32	15.40 ± 0.67	13.85 ± 1.04	13.67 ± 1.17	14.01 ± 1.23	13.00 ± 0.98	14.39 ± 1.28

Analysis of variance for non independent groups - PSC: 1 < 2, 3 ( $p < 0.01$ ); PCC: 1 < 2 ( $p < 0.05$ ).

The other group showed no significant differences.

Analysis of variance for independent groups.

3rd week PSC > PSS, PMM, PMC, PCM ( $p < 0.05$ ).

For the other weeks no significant differences were found.

food consumption by PSC animals, was significantly higher than that of those of groups PSS, PMM, PMC and PCM.

Hair loss and ulcers were observed, mainly at hind paws, independently of the type of diet ingested.

No alteration of hair color of animals which ingested the copper-deficient diet was found.

There was no difference between the groups concerning fertility. All rats were pregnant at term. The number of pregnancy products (fetal resorption, still-borns and live newborns) was not different between the groups as we can see by the mean of each group: PSS = 12.5; PSC = 11.7; PMM = 12.2; PMC = 11.8; PCS = 11.5; PCM = 12.0; PCC = 11.4. The mean of newborns (Table 4), in spite of being numerically smaller in the PMM group, was not significantly different between the groups. This observation was more evident with the lower percentage of live newborns in relation to the total conceptuses in group PMM, as compared to the other groups (Table 4).

The number of newborns with malformations was very small, without predominance in the groups subjected to copper deficiency.

Statistical analysis of the mean weight of litters by group (NBSS = 4.76; NBSC = 4.79; NBMM = 4.81; NBMC = 4.85; NBSCS = 4.67; NBCM = 4.81; NBCC = 4.92), did not show a significant difference.

## DISCUSSION

The initial average weights (90 days) of the studied animals were not different, allowing to affirm the homogeneity of the samples. Despite there being a significant weight gain from the 90th day on, in all groups, it was greater in female rats receiving a moderately copper deficient diet. In spite of controversy in the literature concerning weight gain of animals subjected to deficiency of this trace element, mainly as a consequence of different animal strains and the methodology used, most authors have not observed influence on weight in adult female rats subjected to severe or moderate copper deficiency (17-19). Lower weight gains have been informed in animals subjected to pre- or post-natal deficiency (20, 21), suggesting that copper deficiency influences animal weight when it occurs during its growth and development period.

On the other hand, greater weight gain observed in animals with moderate copper deficiency than in control animals has not been reported in the literature. The explanation for these findings becomes still more difficult with the observation that there was no difference concerning intake, except between moderate and control groups during the 3rd week.

Gradual intake decrease, which occurred in all groups, mainly from the 3rd week on, could be a consequence of a lower dietetic need due to age, or adaptation of the animals to smaller cages, hindering their movements and thus decreasing the energetic requirement. Therefore, intake decrease in groups with copper deficiency does not seem to be related to the ion deficiency, because the same was found in the control group. Other authors who used a similar methodology, also, did not observe this anorexia in animals subjected to copper deficiency. On the other hand, studies with younger animals and for a longer period, notify anorexia starting from the 36th day of the beginning

of the ion deficient diet, this seeming to be directly related to the duration of deficiency (18, 22).

We did not observe great alterations in hair loss or color of the deficient animals. Hair loss in female rats, including the control group, was considered normal for age and adaptation conditions.

Despite the glass bottom cages with openings for passage of feces and urine, decreasing contact of the animals with these materials, it could not be avoided altogether, which we believe, was one of the factors, if not the only one, responsible for the ulcers, mainly at the hind paws. We do not consider involvement of humoral or cellular immunity, described in copper-deficient animals by many authors (21, 23-26), as the cause for these ulcers, since they were also found in the rats of the control group.

Weight gain was not affected significantly during pregnancy in the studied groups, a finding which agrees with the results of other authors (8, 27, 28). The difference of weight gain between PMC and PSS which we found (Table 2), reflects in our opinion, solely a still existing difference, either at the beginning of pregnancy or in the pre-gestational period. Through the comparison of weight gains percentage in the pregnant groups, that did not show significant inter-group difference, we could demonstrate that there was no greater weight gain in PMC rats when compared to those of group PSS (data not shown).

Regarding food intake during pregnancy, our results did not show a strong influence of nutritional copper deficiency, in agreement with other authors (27, 28). Comparison of intake between groups showed that during the 3rd week, animals of group PSC consumed more food than those of groups PSS, PMM, PMC and PCM. It is possible that a larger intake occurred because of an attempt of nutritional recovery to replace copper stores. The fact that we did not observe the same with group PMC may be related to a less deficient diet, to which these animals were subjected, leading, probably, to a lower deficiency and consequently to lower requirements.

According to our results, copper deficiency does not seem to inhibit conception, as also observed by other authors (8, 10, 27, 29). The lower number of newborns observed in group PMM (Table 4) probably is due to a higher number of resorptions found in these animals. These resorptions, however, occurred in different rats, and no total resorption of all conceptuses was appreciated in any animal.

The idea that copper deficiency leads to high incidence of fetal resorptions and stillborns, was reinforced by the works of Hall and Howell (10), and Howell and Hall (29). Nevertheless, these results could not be corroborated by us nor by several other authors who performed more recent work with rats fed before and/or during pregnancy copper-deficient diets (8, 21, 30-32). We are of the opinion that other factors, such as deficiency in other trace elements, could have influenced Hall and Howell's (10) results.

Although the number of fetal resorptions in the PMM group was rather high, we do not consider it to be related to nutritional copper deficiency because the amount of ion per gram of diet received by these animals was four times higher than that of rats of group PSS, wherein the number of resorptions was not significant. Considering that the diet offered to the animals was the same, differing only as to its copper concentration, it is hardly probable that in group PMM there should have been interaction with another element

**TABLE 4**  
**NUMBER OF NEWBORNS (NB) AND RESORPTIONS IN THE DIFFERENT GROUPS STUDIED**

Group	Conceptuses		Total	% NB
	NB	Resorptions		
PSS (1)	116	9	125	92.8
PSC (2)	105	12	117	89.7
PMM (3)	98	24	122	80.3
PMC (4)	108	10	118	91.5
PCS (5)	110	5	115	95.6
PCM (6)	114	6	120	95.0
PCC (7)	107	7	114	93.8
<b>Total</b>	<b>758</b>	<b>73</b>	<b>831</b>	<b>91.22</b>

Chi-square = 24.73 ( $p < 0.01$ ).

Chi-square partition = 21.5 ( $p < 0.01$ ).

3 < 1, 2, 4, 5, 6, 7.

in the diet leading to a higher number of resorptions. Taking into account that this was the only difference between diets, we believe this result to be due to the animal's own condition, and not to the imposed copper deficiency.

Regarding fetal malformations, according to our results, copper deficiency before or during pregnancy does not lead to great fetal abnormalities. Several studies have shown that nutritional copper deficiency, imposed only during pregnancy, does not lead to malformations (8, 33, 34). According to these authors, this is due to the fact that during organogenesis, maternal copper levels are not that low to lead to fetal deficiency. However, when nutritional copper deficiency occurs in the pre-gestational period, most probably fetal malformations will occur. The fact that we did not observe significant malformations in the groups exposed to nutritional copper deficiency before and during pregnancy (PSS and PMM) probably was due to copper plasma levels, not low enough after the first weeks to interfere with fetal organogenesis.

For the analysis of weight of newborns, we used the mean weight for each litter to avoid eventual distortions of the results. Nutritional copper deficiency before or during pregnancy did not alter the weight of newborns, which is in agreement with other authors (8, 27, 34). In two studies wherein the copper-deficient diet was started four months before mating and maintained during pregnancy, the newborns presented normal size and weight at birth, but retarded post-natal growth when compared to controls (32, 35). These findings suggest that consequences of intrauterine copper deficiency are not manifest at birth, but in post-natal life.

In conclusion, not only the intensity of nutritional copper deficiency, but also the time of its occurrence are important for the development of alterations, as a consequence of the decreased ion concentration in the organism.

## RESUMEN

**EFFECTOS DE LA DEFICIENCIA NUTRICIONAL DE COBRE EN RATAS ADULTAS NO PREÑADAS, Y PREÑADAS, Y SUS RECIEN NACIDOS**

Se estudiaron los efectos de la deficiencia nutricional de cobre sobre la ganancia de peso, ingesta de alimento y fertilidad de ratas Wistar adultas, preñadas y sin preñar, y el peso de sus recién nacidos. Los animales adultos fueron sometidos a dietas con diferentes contenidos de cobre (control - 5 ppm; deficiencia moderada - 1.2 ppm; deficiencia severa - 0.3 ppm) durante seis semanas (no preñadas) y nueve semanas (preñadas). No se observó ningún efecto sobre la ganancia ponderal ni sobre el apetito del grupo de animales sujetos a deficiencia de cobre. Ni la fertilidad, evaluada por el número de *conceptus* y nacidos vivos de las ratas preñadas, ni el peso de sus recién nacidos, sufrieron ningún trastorno.

Estos datos revelan que no sólo es el grado de deficiencia, sino también el período durante el cual es impuesto, al igual que su duración, los factores que condicionan la aparición de alteraciones propias de esta deficiencia.

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