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Effect of a leucine-supplemented diet on body composition changes in pregnant rats bearing Walker 256 tumor

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Abstract

Cancer patients present high mobilization of host protein, with a decrease in lean body mass and body fat depletion occurring in parallel to neoplastic growth. Since leucine is one of the principal amino acids used by skeletal muscle for energy, we investigated the changes in body composition of pregnant tumor-bearing rats after a leucine-supplemented diet. Sixty pregnant Wistar rats divided into six groups were fed a normal protein diet (18%, N) or a leucine-supplemented diet (3% L-leucine, L). The pregnant groups were: control (CN), Walker 256 carcinoma-bearing rats (WN), control rats pair-fed with tumor-bearing rats (pfN), leucine-supplemented (CL), leucine-supplemented tumor-bearing (WL), and leucine-supplemented rats pair-fed with tumor-bearing rats (pfL). At the end of pregnancy, all animals were sacrificed and body weight and tumor and fetal weight were determined. The carcasses were then analyzed for water, fat and total, collagen and non-collagen nitrogen content. Carcass weight was reduced in the WN, WL, pfN and pfL groups compared to control. The lean body mass and total carcass nitrogen were reduced in both tumor-bearing groups. Despite tumor growth and a decrease in fetal weight, there was a slight decrease in collagen (7%) and non-collagen nitrogen (8%) in the WL group compared with the WN group which showed a decrease of 8 and 12%, respectively. Although the WL group presented severe tumor growth effects, total carcass nitrogen and non-collagen nitrogen were particularly higher in this leucine-supplemented group compared to the WN group. These data suggest that the leucine-supplemented diet had a beneficial effect, probably attenuating body wasting.

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Malignant neoplasias can kill the host due to fast growth spreading to all tissues and the intense waste of nutrients, or by a combination of these factors (1). Cancer cachexia is characterized by anorexia and host tissue depletion. More than 80% of the patients with malignant disease present features of malnutrition and cachexia as the cause of death (2). During cancer growth the host presents hypercatabolism, mainly of protein, reduced tissue mass and an increasingly negative nitrogen balance. In many cases, the lean body mass of tumor-bearing rats decreases proportionally to the increase of the neoplastic mass (1). A recent experiment has shown increased leucine oxidation in tumor-bear-

ing animals, supporting the demand for both skeletal muscle and tumor tissue, which seems to contribute to the enhanced amino acid turnover (3). In the presence of energy deficiency, tumor cells use other substrates such as alanine, glutamine, lactic acid and ketone bodies (4). Maternal nutrition is very important for the duration of pregnancy. The physiological changes occurring during pregnancy can be sustained by an appropriate nutrient supply to ensure placental and fetal development (5). The association between cancer and pregnancy is about 0.1% (6). Cancer development is difficult to predict and its diagnosis is delayed due to the period, evolution and term of pregnancy (5). In fact, some studies have suggested that pregnancy does not favor the development of cancer, but protects the organism against tumor growth (7). In contrast, recent studies have shown fetal reabsorption and death during the evolution of malignant Walker tumor (8). Protein is intensely mobilized in the organism with cancer, a fact leading to the cachectic state. On the other hand, during the early stages of rat pregnancy, protein is synthesized and accumulated in the maternal organism and is later mobilized during the last days of pregnancy to provide substrates for the fetus (9). To some extent, the fetus can be compared to rapid tumor growth since both tissues have an exponential growth and are dependent on an adequate supply of glucose and amino acids. For this reason, the association between cancer and pregnancy may change the nutritional supply to both tumor and fetal tissues. Furthermore, several studies have reported that the branched chain amino acid leucine independently stimulates skeletal muscle protein synthesis and that enhanced oxidation can prevent alanine release by skeletal muscle (10). Thus, the aim of the present study was to investigate the effect of a leucine-supplemented diet on chemical body composition during tumor growth in pregnant rats.

Young female Wistar rats (45 days old, N = 60) were obtained from the State University of Campinas animal facility, and maintained in the Nutrition and Metabolism Research Laboratory of the Department of Physiology and Biophysics of UNICAMP. Female rats were placed with adult males (90-100 days old, 4 females: one male) in collective cages for one night (12 h) according to the harem method. The first day of pregnancy was determined following detection of sperm in a vaginal smear. Animals were housed in metabolic cages under normal conditions (22 ± 2ºC, 12/12-h light/dark cycle). All animals were fed *ad libitum* a semipurified AIN-G93 diet with free access to drinking water throughout the experiment. Pair-fed non-tumor-bearing controls were offered the same amount of food as that eaten by the tumor-bearing rats. The pregnant animals were divided into six groups and studied for 20 days. Three groups were fed a normal protein diet (18% protein, AIN-G93; N): control rats (CN), tumor-bearing rats (WN), and control pair-fed to tumor-bearing groups (pfN). The other three groups were fed the leucine diet (15% protein and 3% L-leucine, according to AIN-G93; L): control rats fed the leucine-supplemented diet (CL), tumor-bearing rats fed the leucine-supplemented diet (WL), and leucine pair-fed groups (pfL). A Walker 256 tumor cell suspension (approximately 0.25 x 10^6 cells in 0.5 ml of saline) was subcutaneously injected on the right flank of the WN and WL rats, and the control groups (CN, CL, pfN and pfL) were injected with 0.5 ml 0.9% NaCl without anesthesia. The tumor cells were injected immediately after pregnancy was detected. General UKCCR guidelines (1988 United Kingdom Coordinating Committee on Cancer Research) for animal welfare were followed (Ethical Committee for Animal Research, CEEA and COBEA, Brazil, No. 034-2). During the experimental period, changes in body weight, tumor weight, and food intake were recorded every two days. Tumor weight was calculated from 3 orthogonal

linear measurements and compared with the data from a weight dimension curve (8). After 20 days, all rats were killed by cervical dislocation. Placental, fetal and tumor weight were recorded. Gastrointestinal tract, fetuses, placentas, uterine tissues and tumor mass were removed, and all carcasses were kept at -20ºC for chemical analysis of body composition. The amount of carcass water was determined by subtracting dry weight from wet weight. Total fat was extracted with petroleum ether using a soxlet apparatus. An aliquot of the carcass after dry and fat extraction was analyzed for total nitrogen content using a colorimetric micro-Kjeldahl method (11). Another sample was submitted to alkaline digestion with sodium hydroxide plus acidified tannic acid. After these procedures, collagen nitrogen was measured by the colorimetric method described by Albanese and Orto (11). Non-protein nitrogen was calculated by subtracting collagen nitrogen from total nitrogen. Statistical differences between groups were assessed by Kruskal-Wallis one-way analysis of variance (12) followed by the Dunn test, with the level of significance set at $P<0.05$.

At the end of the experiment all pregnant rats presented a gain in body weight compared to that observed at the beginning of the experimental period. Even during pregnancy, when maternal adaptations cause a weight gain, tumor growth induced a reduction in body weight (10% in WN and 15% in WL, $P<0.005$; Table 1) compared to the control groups. These results agree with those obtained in studies on CO/COBS rats bearing Walker 256 tumor for 14 days, which have shown a 20% reduction in food intake and a 15% reduction in body weight (13). In a previous study (8) we detected a 12% body weight reduction in adult pregnant tumor-bearing rats after 21 days. Although the decrease in body weight percentage in both tumor-bearing groups was similar to that reported in other studies, food intake was greatly decreased in these groups (around 32% in the WN and WL groups, data not shown).

Cancer cachexia is a complex syndrome in which, besides fat depletion, the progressive body weight loss occurs mainly because of the wasting of protein tissue (14). Parallel to a greater tumor mass, similar in both tumor-bearing groups independently of diet treatment (Table 1) and a progressive tumor/carcass weight ratio in WN and WL (around 7% in both groups), there was a marked decrease in fetal weight in both tumor-bearing groups (57% in WN and 67% in WL; Table 1). These results also support the idea of a harmful effect of tumor burden especially during pregnancy because tumor implantation caused marked changes in the ratio between placental and

Table 1 - Body and carcass weight, body gain, tumor and fetal weight of the pregnant groups studied.

Data are reported as means ± SEM for 10 animals per group. Groups: control (CN); bearing a Walker 256 carcinoma (WN); control pair-fed to tumor-bearing groups (pfN); control receiving a leucine-supplemented diet (CL); tumor-bearing rats receiving a leucine-supplemented diet (WL), and leucine pair-fed groups (pfL). #Carcass weight represents the total body weight minus placentas, fetus, uterus, intestinal tract and tumor tissue. *P<0.05 compared to control groups; **P<0.05, CN *vs* CL or WN *vs* WL (Kruskal-Wallis test followed by the Dunn test).

	CN	WN	pfN	CL	WL	pfL
Body weight on the first day (g)	140.6 ± 4.92	172.9 ± 7.93*	153.1 ± 2.78	146.2 ± 4.71	162.6 ± 7.56	149.8 ± 5.96
Body weight on the last day (g)	231.0 ± 10.25	221.0 ± 8.01	170.1 ± 8.41*	207.2 ± 8.62	226.4 ± 13.02	146.3 ± 8.20*
Carcass weight (g)#	172.7 ± 2.66	166.1 ± 7.21	140.1 ± 6.35*	160.1 ± 3.14	165.4 ± 9.34	120.0 ± 6.81*
Body weight gain on the last day (%)	165.5 ± 8.55	128.9 ± 4.52*	111.8 ± 6.53*	141.9 ± 5.06**	135.2 ± 7.03**	98.18 ± 5.72*
Tumor weight (g)		11.73 ± 0.93			11.58 ± 0.68	
Fetal weight (g)	3.73 ± 0.13	1.61 ± 0.22*	2.98 ± 0.34*	3.45 ± 0.16	1.15 ± 0.16	2.77 ± 0.28*

fetal weight. In the WN group, the fetal weight decrease was positively correlated with the placental weight decrease ($P<0.01$; data not shown) and therefore the nutritional support to fetal growth could be due to reduced placental function. This idea is supported by the fact that fetal weight reduction was less intense during nutritional deficiency, such as that observed in both pfN and pfL groups (a 20% fetal weight decrease). In addition, the leucine-supplemented diet associated with tumor growth induced a reduction in fetal and placental weight similar to that observed in the WN group.

Despite the reduction in fetal weight induced by the leucine-enriched diet, there was no correlation between the placental and fetal weight, suggesting that other mechanisms may be involved in the harm to the maternal unit. Previous studies have shown that tumor implantation reduces placental and fetal weight and protein content in the placenta and fetus of adult tumor-bearing rats (8), and produces intensive hemorrhage and edema in the placenta of tumor-bearing rats (15). In contrast, the implantation of Yoshida AH-130 ascites hepatoma during the last week of rat gestation resulted in normal fetal growth (16). In most studies, the percentage of carcass water was higher in cancer patients (17). The body water content was slightly increased in the WN, WL (6% and 7%, respectively) and pair-fed groups (6% in pfN and 9% in pfL). Therefore, our data suggest that the changes produced by tumor growth in these pregnant rats do not support the alterations in body water content observed in cancer patients and in animals (18). The fat carcass decrease observed in both tumor-bearing groups (32% in WN and 20% in WL) may have been due to the intense fat mobilization from adipose tissue especially in the WN group.

Depletion of host fat stores is a common finding in cancer cachexia. In an extensive review (14), this loss of body fat was related, in part, to a circulatory lipid-mobilizing factor derived from the tumor cells. Although anorexia is common in cancer, loss of carcass fat cannot be attributed to a decreased calorie intake alone, since pair-fed animals do not lose as much fat as tumor-bearing rats. In contrast, we observed that the decrease in body fat was similar in both tumor-bearing and pair-fed rats. This was possibly related to the catabolic phase during the last days of the rat pregnancy. In pregnant rats, body fat accumulation reaches a maximum at 19 days of gestation and declines on the 21st day, but several fat depot changes are highly dependent on the animals' feeding condition, since they change in magnitude and even in direction in the fasting state (19). A slight decrease in lean body mass, which was not significant, was observed in the WN group (Table 2).

Other studies have shown a significant increase in proteolysis in cancer patients, twice as high as observed in malnourished patients. This wasting of lean body mass reveals the incapacity to conserve body protein (20). In contrast, the WL group presented a slight increase in lean body mass compared with its respective control group. However, the increase in WL lean body mass was significantly higher than in the WN group (around 12%), suggesting an improved effect of the leucine-enriched diet. In spite of a slight (not significant) decrease in total carcass nitrogen in both tumor-bearing groups (Table 2), the WL group kept the total nitrogen level (13% higher than in the WN group) even during the wasting of tumor growth. Collagen nitrogen represents the extracellular mass that can be less metabolically active and less wasted than the cellular mass. The collagen nitrogen was preserved in the WN group compared to the WL group, which presented a significant decrease in this parameter compared to the CL group (Table 2).

Carbo et al. (16) suggested that pregnancy could protect against the cachexia induced by the tumor or retard its appearance. The authors found a similar increase in

Table 2 - Body water content, body fat, lean body mass, total carcass nitrogen, and collagen and non-collagen nitrogen content of the pregnant groups studied.

Data are reported as means ± SEM for 10 animals per group. See Table 1 for group abbreviations. *P<0.05 compared to the respective control groups; **P<0.05 compared to the WN group (Kruskal-Wallis test followed by the Dunn test).

	CN	WN	pfN	CL	WL	pfL
Body water (%)	56.81 ± 1.46	60.85 ± 1.73	59.39 ± 0.58	56.79 ± 2.55	59.46 ± 1.46	61.31 ± 0.54
Body fat (%)	13.49 ± 0.68	9.10 ± 1.79*	8.07 ± 0.87*	15.07 ± 2.17	12.07 ± 1.62	5.56 ± 0.42*
Lean body mass (g)	39.54 ± 0.86	37.71 ± 1.46	37.53 ± 1.44*	39.24 ± 0.90	42.22 ± 1.45**	33.05 ± 1.93*
Total nitrogen (mg/100 g carcass weight)	65.61 ± 3.10	60.29 ± 3.58*	63.90 ± 4.32	69.74 ± 4.10	68.27 ± 3.47**	54.21 ± 1.70*
Collagen nitrogen (mg/100 g carcass weight)	5.68 ± 0.53	6.96 ± 0.42*	7.08 ± 0.50	8.57 ± 0.55*	6.82 ± 0.77*	6.48 ± 0.75
Non-collagen nitrogen (mg/100 g carcass weight)	59.82 ± 3.10	53.33 ± 4.21*	56.87 ± 4.49	61.17 ± 3.89	60.27 ± 4.34**	47.73 ± 1.71*

proteolytic rates in both tumor-bearing pregnant and virgin rats, but in spite of a great reduction in both protein synthesis and degradation in the tumor-bearing groups, these changes were similar to those observed in the control pregnant groups. Cohn et al. (17) reported that non-collagen nitrogen represents the cellular mass and during pathological states there is a preferential mobilization of this protein store.

In the present study, we found a significant reduction in non-collagen nitrogen only in the WN tumor-bearing group (around 11%; Table 2). In contrast, WL rats presented a 13% higher non-collagen nitrogen level than WN, suggesting that the waste in body nitrogen was lower in the leucine-supplemented group. Although collagen nitrogen was decreased in the WL group, non-collagen nitrogen was maintained in these animals, suggesting that leucine supplementation provided a substrate to preserve the nitrogen carcass. Peripheral muscle wasting may be due to increased muscle catabolism, decreased protein synthesis, or a combination of the two (14). While there appears to be an overall decrease in the synthesis of muscle protein in cancer patients, there is an increase in whole-body protein turnover leading to an elevated endogenous protein breakdown and oxidation of amino acids. In addition, amino acid requirements are altered in the tumor-bearing state. Host leucine requirements have been shown to be increased in the presence of a rapidly growing tumor. Some malignant cells have specific requirements for essential amino acids. Removal of one amino acid by the tumor would lead to a depression of host protein synthesis (14). For this reason, since normal synthesis requires the full complement of amino acids, we may suggest that supplementation with one amino acid (for example, leucine) probably supported the high rates of muscle oxidation, contributing to the decreased output of some amino acids from skeletal muscle, which could be used by neoplastic cells. Further studies are currently underway to find out whether leucine supplementation could be of benefit to the host carcass by reducing peripheral tissue mobilization.

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