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ORIGINAL CONTRIBUTION

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Nutritional treatment of cancer cachexia in rats

Use of a diet formulated with a crayfish enzymatic extract

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Abstract *Background* Terminal cancer-associated cachexia, characterized by a marked weight loss, anorexia, asthenia and anemia, is usually associated with a malnutrition status. *Aim of the study* To investigate whether a diet formulated with a crayfish enzymatic extract, enriched in essential amino acids, omega-3 fatty acids, and astaxanthin, would be effective for the treatment of cancer-associated cachexias, by decreasing mortality and morbidity rates in cachectic rats and/or improving survival. *Methods* Two types of diet were used: a standard diet and one formulated with crayfish enzymatic extract. Rats were divided into two groups (24 animals per group): one without tumor (T−) and the other with tumor (T+) (AH-130 Yoshida ascites hepatoma). Each group was further divided into two subgroups (12 animals per subgroup). Two subgroups ($T-_{standard}$ and $T+_{standard}$) were fed the standard diet and the other two ($T-_{CFEE}$ and $T+_{CFEE}$) the crayfish enzymatic extract one for four weeks, after which different tissue and plasma parameters were studied. *Results* The implantation of the tumor resulted in a considerable loss of muscle and adipose tissue mass in both groups, but the loss of muscle and fat was lower in the group fed the crayfish enzymatic extract diet. There was also a concomitant increase in the plasma concentration of TNF-α, although the increase was smaller in the crayfish enzymatic extract-treated group. *Conclusion* This study shows that although the treatment of cachetic rats with the crayfish enzymatic extract diet did not revert the cachexia, it increased survival (57.1% vs. 25.9% in the group treated with crayfish enzymatic extract and standard diets, respectively) and meliorated the cachexia symptoms – anorexia and body mass loss (muscle and adipose tissue).

Key words crayfish – enzymatic extract – cancer – cachexia – omega-3 fatty acids – astaxanthin

Introduction

Muscle wasting is a major characteristic of the cachexia associated with diverse pathologies such as terminal cancers, bacterial sepsis, and AIDS. Cachexia occurs in the majority of terminal cancer patients, and can contribute substantially to morbidity and mortality, as well as affecting seriously the quality of life and causing distress to patients and their families [9].

According to recent findings, the progressive weight loss and muscle wasting seen in cancer patients can be halted or even reversed by giving them a high-energy, and high-protein supplement containing omega-3 fatty acids, in particular EPA [27]. Therefore, in the development and evaluation of new nutritional therapies for the treatment of patients with terminal cancer-associated cachexia, it would be advantageous to use a readily available low-cost and non-toxic product (with high nutritional value, and protective effects) that improved weight loss and reduced the production and release of inflammatory mediators and oxygen active substances and/or free radicals.

To support this effort, various experimental and clinical studies have proved the benefit of consuming a diet supplemented with high-quality proteins (rich in essential amino acids) [21], omega-3 fatty acids [3, 7], and antioxidants [10]. Until now, except in the case of the group of Fearon and Barber [4] who used a pharmaco-nutritional support with energy dense and high protein content supplemented with eicosapentaenoic and docosaexaenoic acid, and the recent papers by Mantovani et al. [20] and by Cerchietti and Navigante [8], few experiments have been carried out supplementing the diet with a natural product containing all three beneficial components: high-quality protein (rich in essential amino acids), omega-3 fatty acids, and antioxidants.

Crayfish enzymatic extract, obtained from crayfish (*Procambarus clarkii*) [12] is a product containing high-quality proteins, characterized by its high contents in essential amino acids, omega-3 fatty acids and carotenoids (mainly astaxanthin). Because of its unique composition, we used crayfish enzymatic extract for the treatment of rats bearing the Yoshida AH-130 ascites hepatoma – a very cachectic rat tumor model that results in a high concentration of circulating pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, etc. [11], where high-quality nutrients are required.

The present work shows results on crayfish enzymatic extract use for the treatment of cancer-associated cachexia in a rat model, following the evolution of cachexia parameters, such as weight, muscle and adipose mass, anorexia and pro-inflammatory cytokine levels (TNF-α).

Materials and methods

Animals and diets

Male Wistar rats (Seville University Animal House), weighing about 100 g, were used. The animals were maintained on a regular light/dark cycle (12 h light, 12 h dark) with free access to food and water.

Table 1 Composition in grams per kilogram (g/kg) of standard and crayfish enzymatic extract diet

	Standard diet	Crayfish enzymatic extract diet
Protein (12%)		
Casein[a]	133.3	–
CFEE[b]	–	166.0
Fat (5%)		
Olive-oil	39.3	33.7
Carbohydrate (63.5%)		
Corn starch	150.0	120.8
Sucrose	485.0	485.0
Methionine	3.0	3.0
Vitamin mix[d]	1.0	1.0
Salt mix[e]	3.5	3.5
Choline bitartrate	2.0	2.0
Cellulose	182.9	185.0

CFEE: crayfish enzymatic extract
[a](Casein: 90% protein, 8% fat)
[b](CFEE: 72.3% protein, 9.8% fat, 17.6% carbohydrate)
[c]CF: crayfish
[d]AIN-76AVitamin mixture
[e]AIN-76 Mineral mixture

Two types of diet were used: (i) the standard diet, consisting of 63.5% carbohydrate, 12% protein (casein) and 5% fat (the difference to 100% comprised minerals, vitamins and non-digestible material, mainly cellulose); (ii) the crayfish enzymatic extract diet, an isocaloric and isoproteinic diet formulated with crayfish enzymatic extract (see Table 1).

Diets were prepared every 2 weeks and frozen at −20°C; aliquots were thawed at room temperature and used on alternate days. All rats were fed the diet and water ad libitum. Food intake was measured daily using a metabolic cage (Tecniplast, 3700M071). The protocol used in this study was approved by the Ethics Committee for Animal Experimentation of Seville University (Spain), based on the recommendations of the European Council (EC 806/89).

Crayfish enzymatic extract composition

Crayfish enzymatic extract was prepared by autodigestion according to the procedure we have described previously [12, 13]. The production process was carried out, with minor modifications, under aseptic conditions, and the final product was lyophilized instead of being dried in a spray-dryer. Chemical characterization of this product shows that the crayfish enzymatic extract has a significant content in protein (72.3 ± 5.1%), fat (9.8 ± 0.8%) and total carotenoids (612.6 ± 47.0 μg/g) content. The main feature of crayfish enzymatic extract protein is its amino acid composition, containing all the essential amino acids. Such composition meets all the FAO requirements for nutritional purposes [14]. The high content in essential amino acids (46.60 ± 1.4% of the

total amino acid content), which represent 55.9 ± 3.9 g essential amino acids/kg diet, and in ω-fatty acids (30.7 ± 1.9% of total fat), which represent 5.0 ± 0.3 g/kg diet, together with its content in carotenoids (mainly astaxanthin), makes this product an important protein source for the nutrition of patients needing protein of high quality (rather than in large amounts) and/or an antioxidant source, as in the case of cancer, AIDS, hepatic, and renal patients, and in the elderly [27].

Tumor inoculation

The tumor host group received an intraperitoneal inoculum of 10^7 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumours (approx. 2 ml), and the control rats were injected with 2 ml of 0.9% (w/v) NaCl solution. The Yoshida AH-130 is a rapidly growing tumor with a volume doubling time of 1 day [29]. Total cell content was estimated using Trypan blue staining.

Experimental design

Rats were divided into two groups (24 animals per group): one without tumor (T−) and the other with tumor (T+). Each group was further divided into two subgroups (12 animals per subgroup): two subgroups were fed the standard diet: $T-_{standard}$ and $T+_{standard}$ and the other two the crayfish enzymatic extract one: $T-_{CFEE}$ and $T+_{CFEE}$, for 4 weeks, after which different tissue parameters (muscle and adipose mass) and plasma TNF-α level were studied. Tissues were rapidly excised, weighed and frozen in liquid nitrogen.

Analytical assays

Total nitrogen and total fats were determined according to the standard methods of the AOAC [1]. Protein concentration was determined by a high-performance liquid chromatography (HPLC) amino acid analysis after hydrolysis with 6N HCl in the presence of phenol and under vacuum atmosphere at 105°C for 18 h. The amino acid composition of the hydrolyzed fractions was determined by a reversed-phase HPLC analysis using the method of Bidlingmeyer et al. [5]. Cystine and methionine were determined by performic acid oxidation [23] and tryptophan by basic acid hydrolysis [16]. Nitrogen content of chitin was estimated by the Kjeldahl method, after the sample had been purified of its calcium carbonate and protein by boiling with acid and alkali respectively [6]. Chitin was calculated by multiplying chitin nitrogen by 14.5, assuming that the pure chitin contained 6.9% nitrogen [25].

Fatty acid methyl esters were quantified by capillary gas chromatography with flame ionization detection [19].

Total carotenoids were extracted with diethyl ether, and a quantitative determination was made spectrophotometrically at 474 nm according to the procedure described by Negro and Garrido-Fernandez [24], using an extinction coefficient of $E^{1\%}{}_{1\ cm} = 1{,}900$ [28].

TNF-α concentration in arterial blood was determined using a standard sandwich-ELISA for rat TNF-α (Biosource International, California, USA). A standard curve using the recombinant cytokine was constructed to quantify the cytokine.

Statistical analysis

Results are expressed as the mean ± S.D., and comparison of several rats groups, were done by one-way ANOVA followed by Bonferroni post hoc multiple comparisons test. The significance was set at 95% of confidence. Significant differences are referenced as $P < 0.05$ in the text.

Survival studies

Rat's mortality data were compared using Kaplan–Meier plots and Logrank tests [17].

Results

Evolution of animal weight and food intake

Figure 1 shows that tumor growth led to a marked decrease in food intake, observed even in the first 7 days. However, this decrease is statistically significant lower ($P < 0.05$) for the group feed the crayfish enzymatic extract. During the first 7 days of the experiment no significant difference in weight was observed between the two tumor-bearing groups; however, the difference was statistically significant with respect to the corresponding non-tumor bearing group. Figure 2 shows the weight evolution for the groups of rats without tumor and rats with tumor during 4 weeks. The growth curve of the rats without tumor increases progressively during the 28 days of assay, while the curve of the tumor-bearing rats reaches a plateau at 12–14 days after tumor implantation. However, at the end of the experiment, it was statistically significant ($P < 0.05$).

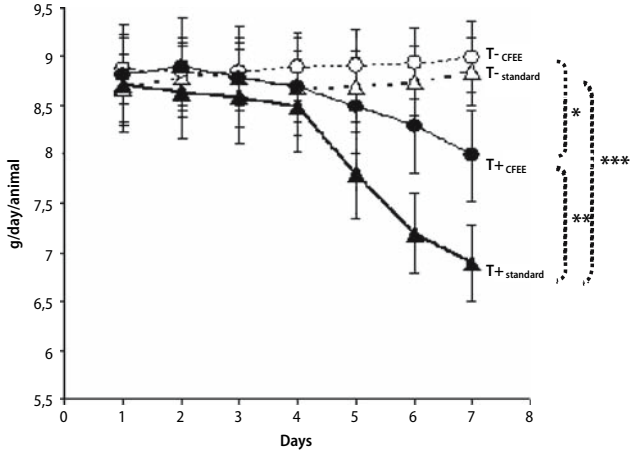

Fig. 1 Food intake in the four groups of rats: $T-_{standard}$ (standard diet without tumor), $T-_{CFEE}$ (crayfish enzymatic extract diet without tumor) $T+_{standard}$ (standard diet with tumor) and $T+_{CFEE}$ (crayfish enzymatic extract diet with tumor). CFEE: crayfish enzymatic extract. Experimental period of 7 days, and 12 animals studied per group. *$P < 0.05$, **$P < 0.05$, ***$P < 0.05$

Survival

After 28 days of experiment, the rate of surviving animals in the group with tumor was, 57.1 and 25.9% for the animals fed the crayfish enzymatic extract and standard diet, respectively. Although at 45 days all the animals treated with the standard diet were dead, three animals (14.3%) treated with the CFEE diet survived until 56 days. These data shows that mortality is influenced by the diet treatment strategy. Intergroup comparisons (Kaplan–Meier, Logrank test) show a statistically significant difference ($P < 0.001$) (See Fig. 3).

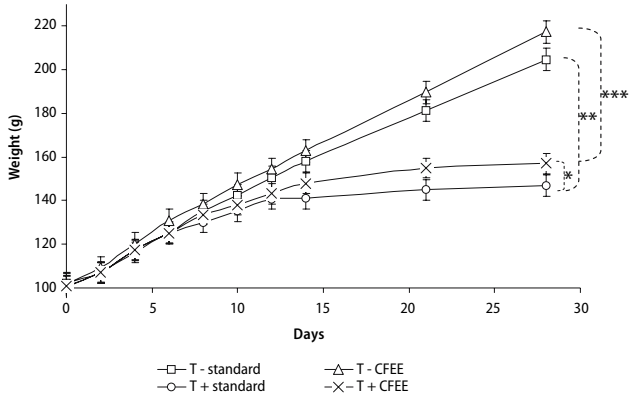

Fig. 2 Growth curves of the four groups of rats: $T-_{standard}$ (standard diet without tumor), $T-_{CFEE}$ (crayfish enzymatic extract diet without tumor) $T+_{standard}$ (standard diet with tumor) and $T+_{CFEE}$ (crayfish enzymatic extract diet with tumor). CFEE: crayfish enzymatic extract. Experimental period 28 days. Number of animals per group: 12 respectively. *$P < 0.05$, **$P < 0.05$, ***$P < 0.05$

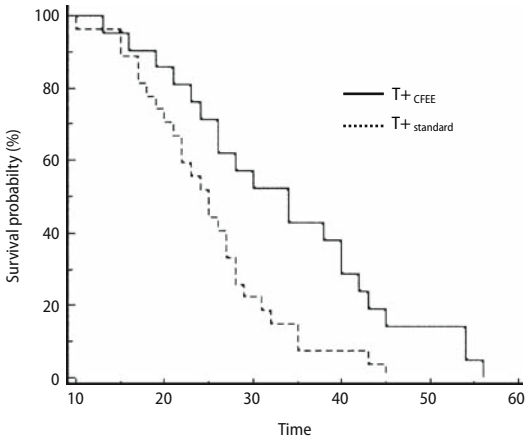

Fig. 3 Kaplan–Meyer survival curve in the two groups: $T+_{standard}$ (standard diet with tumor) and $T+_{CFEE}$ (crayfish enzymatic extract diet with tumor). Experimental period 56 days. Number of animals per group: 21 and 27 respectively. Comparison of survival curves (Logrank test): $P < 0.01$

TNF-α plasma level

The level of plasma TNF-α in the CFEE-treated group was lower than in the one treated with the standard diet: 49.7 ± 5.2 and 59.9 ± 6.9 pg/ml, respectively.

The level of plasma TNF-α in the healthy controls was 17.8 ± 1.2 pg/ml in the $T-_{standard}$ group and 18.2 ± 1.6 in the $T-_{CFEE}$. These values were significantly lower than that observed in the tumor bearing groups, 59.9 ± 6.9 pg/ml, in the group treated with the standard diet and 49.7 ± 5.2 in the one treated with the crayfish enzymatic extract; however the difference of plasma TNF-α level between both tumor bearing groups is statistically significant ($P < 0.05$).

Adipose and skeletal muscle weight

As shown in Fig. 4a, tumor-bearing rats fed the standard diet underwent a marked decrease in the weights of *tibialis*, *gastrocnemius*, *soleus*, and *vastus* muscles as compared with the respective non-tumor-bearing control. Although, these decreases were less pronounced in the group of tumor-bearing rats fed the crawfish enzymatic extract diet, as compared with the non-tumor-bearing control group. However, the differences in the weights of these muscles, between both tumor-bearing groups, are also statistically significant ($P < 0.05$).

In tumor-bearing rats fed the standard diet, tumor growth also caused a substantial decrease in adipose mass (Fig. 4b), i.e., in white dorsal, white peritoneal, and brown interscapular adipose tissue. As in the case of muscle, here the group of rats fed the CFEE diet also showed a less pronounced effect. In this case, the differences in weights of adipose tissues, between

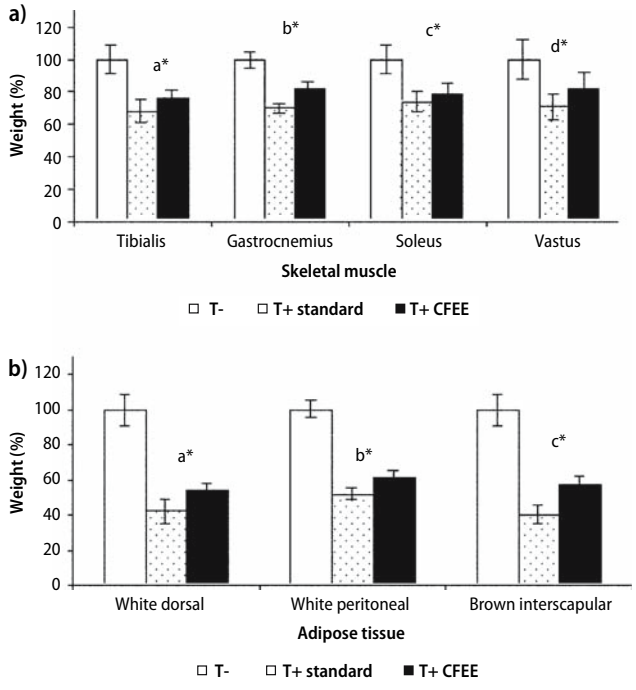

Fig. 4 (**a**) Skeletal muscles weight in the three groups of rats: T− (standard diet without tumor), $T+_{standard}$ (standard diet with tumor) and $T+_{CFEE}$ (crayfish enzymatic extract diet with tumor). CFEE: crayfish enzymatic extract. Experimental period 28 days. Number of animals per group: 12 respectively. a,b,c,d: $T+_{CFEE}$ vs. $T+_{standard}$; $^*P < 0.05$. (**b**) Adipose tissue weight in the three groups of rats: T− (standard diet without tumor), $T+_{standard}$ (standard diet with tumor) and $T+_{CFEE}$ (crayfish enzymatic extract diet with tumor). CFEE: crayfish enzymatic extract. Experimental period 28 days. Number of animals per group: 12 respectively. a,b,c,: $T+_{CFEE}$ vs. $T+_{standard}$; $^*P < 0.05$

both tumor-bearing groups, is also statistically significant ($P < 0.05$).

Discussion

As our results show, tumor growth led to a marked decrease in food intake, observed even in the first 7 days, which was without doubt partially responsible for the decrease in body weight observed in the tumor-bearing animals. These results are in agreement with studies of other groups [11, 22, 27]. Marked reduction of food intake is associated with a concomitant reduction of muscle and adipose tissues.

Administration of crayfish enzymatic extract diet did not revert the anorexia and the decrease in body weight. Although it partly controlled the anorexia, it had a less pronounced effect on total body weigh, probably, because the changes in the plateau zone shown by tumor-bearing animals are less pronounced or significant at this stage of the illness.

Although food intake decreased in both tumor-bearing groups, this was less pronounced in the crayfish enzymatic extract diet fed group than in the group fed the standard diet (11.3 ± 0.5 and 22.0 ± 0.9% respectively). These results can be interpreted as an amelioration of the cachectic process in the group fed crayfish enzymatic extract diet, due to a better diet.

The growth curve of the rats without tumor increased progressively during the 28 days of assay, while the curve of the tumor-bearing rats reached a plateau at 12–14 days after tumor implantation, probably as a consequence of metabolic alterations caused by the tumor [15, 26] leading to a predominance of catabolic processes over anabolic ones, and to a lower food intake, as will be discussed later. Comparison of the growth curves of the two tumor-bearing groups, treated with standard and crayfish enzymatic extract diet, shows that although the body weight reached a plateau at mid-assay in both cases, the evolution of body weight a long the complete experiment was statistically different ($P < 0.05$), although it was not as greater as expected. Growth of the crayfish enzymatic extract-treated group was higher than that of the standard-treated group. We attribute this difference to the synergistic action of all the crayfish enzymatic extract diet components, high content of essential amino acids (55.9 ± 3.9 g/kg diet), omega-3 fatty acids (1.8 ± 0.1 g/kg diet) and the antioxidant astaxanthin (101.7 ± 7.8 mg/kg diet), mainly. Similar effects has been also described for diet supplemented with high-quality proteins (rich in essential amino acids) [21], omega-3 fatty acids [3, 7], and antioxidants [10].

These results are in accordance with the data reported by others [22, 27] for similar products such as pharmaco-nutritional support with energy dense and high protein content supplemented with eicosapentaenoic and docosaexaenoic acid [4].

Tumor burden also resulted in a marked reduction in adipose tissue, together with a clear hyperlipemia. The loss of fat mass is the result of several altered processes: (i) an increase in lipolytic activity, which results in a significant release of both glycerol and fatty acids [2], and (ii) a marked decrease in the activity of lipoprotein lipase (LPL). This enzyme is responsible for the cleavage of both endogenous and exogenous triacylglycerols (present in lipoproteins) to form glycerol and fatty acids (in white adipose tissue) [15], which is reflected as hypertriglyceridemia, partly due to a decreased lipoprotein-lipase activity in adipose tissue [15]. The smaller decreases of muscle and adipose tissues observed in tumor-bearing rats treated with the crayfish enzymatic extract diet cannot be due only, to the smaller?increase of TNF-α plasma level observed in this group; rather it might be due to

the reduction in the levels of other cytokines (not studied in this work) which are released by the tumour and possibly contribute with TNF-α to the induction of cachexia.

Some pathological processes, such as many cancers, get worse due to certain irregularities of the immune system, which are characterized by an uncontrolled production and/or release of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, etc. In the tumor-bearing rats, although nutritional treatment with the crayfish enzymatic extract diet did not reduce the level of plasma TNF-α to that in non-tumor-bearing animals, the level of plasma TNF-α in the crayfish enzymatic extract -treated group was lower than in the one treated with the standard diet. TNF-α, previously called cachectin, has been implicated in mediating cachexia [26], and it has been shown to decrease LPL activity in adipose tissue and to inhibit glucose transport in adipocytes, so that the availability of substrates for lipogenesis decreases [2]. TNF-α also seems to have an important role in the loss of proteins from skeletal muscle of tumor-bearing animals, as has been reported for tumor-bearing transgenic mice, which overexpress the soluble TNF receptor-1 [18]. Although TNF is only one of a number of pro-inflammatory cytokines thought to be responsible for the physiological changes of cachexia, the observed differences in TNF may reflect a modulation of the pro-inflammatory state leading to the other changes observed. These results may be important, because the increased level of plasma TNF-α could be the origin of other physiological and metabolic disorders, such as hyperlipidemia, increase of hepatic gluconeogenesis, and decreases in adipose and skeletal muscle mass [22]. Therefore, these data could also explain the smaller decrease in muscle and adipose mass observed in the group fed the crayfish enzymatic extract diet, as will be shown below. Although the treatment with the crayfish enzymatic extract diet was unable to reverse any of the changes associated with cachexia, such as muscle- and lipid-mass loss, it did induce a statistically significant decrease in the level of plasma TNF-α in tumor-bearing animals treated with the crayfish enzymatic extract diet. Thus, the treatment with crayfish enzymatic extract diet for 4 weeks decreased the production of pro-cachectic cytokines, although cachexia is not reverted.

The most important result of this experiment is the significant difference in survival between the tumor-bearing group treated with the crayfish enzymatic extract diet and the tumor-bearing one treated with the standard diet (57.1 vs. 25.9% respectively) at the end of the assay (28 days). Another important observation is that at 45 days, all the animals treated with the standard diet were dead, but 14.3% of the animals treated with the crayfish enzymatic extract diet survived until 56 days.

In conclusion, although the crayfish enzymatic extract diet do not revert cancer-mediated cachexia, it can increase the survival of tumor-bearing animals and meliorate the cachexia symptoms – anorexia and body mass loss (muscle and adipose tissue).

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