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Nucleotides and Effect Over Starving Condition on Fish SHK-1 Cells Model

Pamela Olivares[1], Rodrigo Sanchez[1,4], Erico Carmona[2], Allisson Astuya[3], Hector Herrera[4] and Jorge Parodi[1]*

[1]*Laboratorio Fisiología de la Reproducción, Escuela de Medicina Veterinaria, Núcleo de Investigación en Producción Alimentaria, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile*
[2]*Grupo de Genotoxicología, Escuela de Medicina Veterinaria, Núcleo de Estudios Ambientales, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile*
[3]*Laboratory of Cell Culture and Marine Genomics, Marine Biotechnology Unit, Faculty of Natural and Oceanographic Sciences, University of Concepcion and Sur-Austral COPAS Program, University of Concepcion, Casilla 160-C, Concepción, Chile*
[4]*Empresa Vitapro Chile, Castro, Región de los lagos, Chile*

Abstract

Additives play an important role in nutrition for boosting the physiological processes of productive species, and starvation has become a new problem for the industry. Only in recent years have studies began to analyze the effects of additives on fish cells. We observed the effects of nucleotides, which is a compound highly utilized in aquaculture. The SHK-1 cell line was derived from the head and kidney of Atlantic salmon as a biological model. Samples were exposed to nucleotides at incremental times and concentrations to determine the effects on cell viability by evaluating the number of cells. The cells were also exposed to a starving condition and recovery when nucleotides were used. Our data indicate that nucleotides are not sufficient to stimulate cellular proliferation. Cultures were exposed to a basal medium without serum in the presence of or absence of nucleotides. We observed that the starving effects are reduced when the basal medium is supplemented with nucleotides. Our results indicate that it is important to evaluate additive effects at a cellular level and that nucleotides have nutritional effects when the cells are in the cellular starving condition. Our study helps to generate more rational applications of additives in the industry and presents new challenges.

Keywords: Fish; Nutrition; Nucleotides; Cell cultures

Introduction

In the industry of productive species, additives play an important role in boosting physiological processes. Nucleotides, which are precursors of DNA replication, have long been recognized as important elements in mammalian nutrition [1]. Regarding resistance to infections, it has been shown that groups of mice that are fed diets supplemented with nucleotides have less mortality following infection with *Staphylococcus aureus* [2] and *Candida albicans* [3] than groups of mice that are fed nucleotide-free diets.

This heightened resistance to infection is reported to be the result of increased phagocytic activity of murine peritoneal macrophages [2], increased T-cell dependent antibody production [4], and enhanced and elevate bone marrow cells and increase peripheral neutrophil numbers [5]. But at cellular level the mechanism of the nucleotides in fish are complete described. We don't have evidence of physiological roll of the nucleotides in the fish cells and the biotechnology offer opportunity for initiative this kind of study.

Cellular cultures are important for fish research and in vertebrates, as well as for the exploration of additives and their interaction with organs and the appearance of secondary effects [6]. Cell cultures also provide unlimited biological material for diagnosing alterations that are produced by additives [7,8]. Cell lines are the study models for molecular effects on cell function [9].

In this study, SHK-1 cells derived from the frontal head and kidney of Atlantic salmon were treated at for several days and different nucleotide concentrations, when cells are in the presence of absence of the complete culture medium. Then, the number of cells in response to proliferation and their viability in the starving condition were analyzed, for help to suggest the used of these additives on salmon industry.

Materials and Methods

Cellular cultures

Cell lines derived from leucocytes in *Salmo salar* SHK-1 (ECACC N° 97111106) were maintained in an incubator at 17°C. Cells were cultivated in a Leibowitz L-15 medium (Life Technologies) and supplemented with 10% fetal bovine serum (Life Technologies), L-glutamine 1%, penicillin/streptomycin 1% (Life Technologies) and β-mercaptoethanol 7.2 µL/ml. The cellular expansion procedures were carried out in a biosafety cabinet and were seeded in plates of 24 wells for a parallel experiment. For maintenance and subculture, the medium was changed every 3 days. Cultures were exposed to temporal curves (one to seven days) and concentrations (0.1 to 1000 ppm) of generic nucleotides obtained from the Nucleoforces Salmonids™ Mix, which are commercial additives used in the formulation of a fish diet. Staining and morphology were analyzed to explore the reaction to the additive. We modified a study by [10] and created the starving model using only a basal medium without serum to generate a poor growth condition, and it was used for seven days to generate a starving condition.

Cellular proliferation

Cells were washed with Phosphate buffer solution (PBS) and removed with a trypsin solution 1%, after which they were placed in a complete culture medium to inhibit the trypsin. Samples were collected in sterile 15-ml tubes and were centrifuged at 1200 rpm for 10 minutes. Cells at the bottom were suspended in 1 ml of base medium, and a

***Corresponding author:** Jorge Parodi, Av. Las Mariposas S/N, Campus Dr. Rivas, Laboratorio Fisiología de la Reproducción, Escuela de Medicina Veterinaria, Núcleo de Investigación en Producción Alimentaria, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile, Tel: +5642205564; E-mail: jparodi@uct.cl

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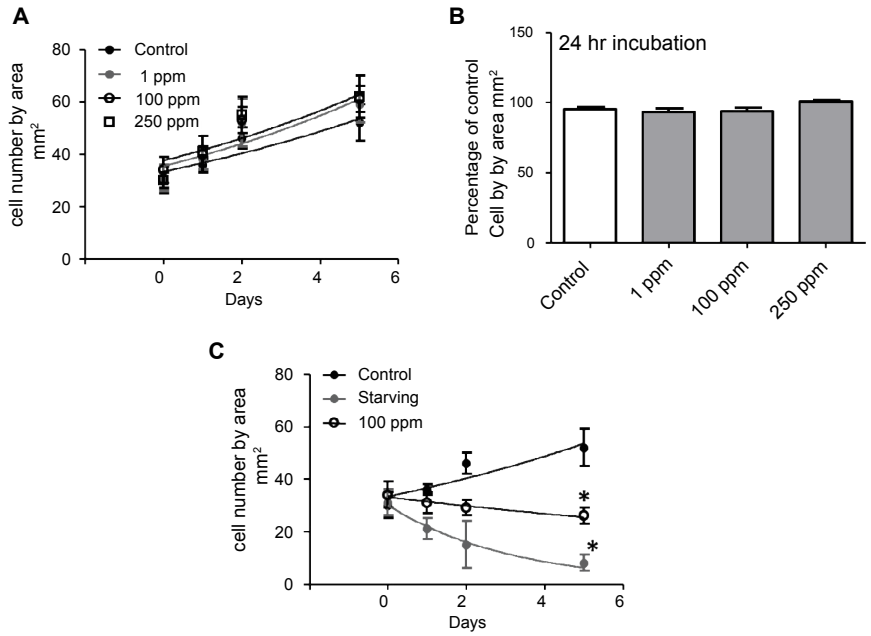

Figure 1: Effect on cellular starving (A): the number of cells after 5 days of culture in the absence or presence of nucleotides. (B): number of cells of the culture exposed to different concentrations of nucleotides after 24 hours. (C): number of cells after 5 days of culture in the absence of serum, which is the starving condition, and in the absence or presence of nucleotides. Each bar or point represents the mean ± SEM of the measurements of at least 5 independent cultures and experiments. The asterisk indicates $p<0.05$ (ANOVA).

sample was taken to be recounted in a haemocytometer that was diluted 1/100. With this value, samples were seeded at a density of 100,000 cells per ml in a culture with a 1.6-mm diameter and 250 µl of medium. This culture was exposed to the additives for 24 hours, and the number of cells was counted based on area. The density of the cells was recorded as an indicator of the change in cellular proliferation or growth rate, which was modified from Parodi et al. [11].

May-Grunwald and Giemsa staining

Cells grown in culture plates were exposed to temporal curves and concentrations of additives and were washed of the culture medium and incubated with a PanOptic kit for May-Grunwald and Giemsa staining (Quimica clinica aplicada, Spain). Samples were left for 5 seconds in reactive 1, 10 seconds in the May-Grunwald reactive and 10 seconds in the Giemsa reactive. The plates were washed with ultrapure water and left to be microphotographed with a NIKON Labophot 2 optic microscope and a 519CU 5.OM CMOS camera, which was modified from Flores et al. [12].

Data analysis

We used the Shapiro-Wilk test, which is a test for normality, to determine how far the distribution was from a Gaussian distribution. The results that include an image analysis are presented using the average ± SEM. We used Prism 5 software for the analysis. Statistical comparisons were carried out using a two-way analysis of variance (ANOVA), as indicated in the figure legend, and the Bonferroni test was used after the analysis (Post-test). A probability value of $p<0.05$ is considered statistically significant.

Results

The cells were seeded, and the beginning of the experiment is time=0 (initial condition). Figure 1A shows the growth curves of cells in the control condition and cultures that were treated with 10 ppm, 100 ppm and 250 ppm of nucleotide from time=0 and at 5 days of exposure. The results demonstrate an increased number of cells per area in the exposed sample. Figure 1B shows the cell percentage per area (mm^2) at 24 hours of incubation in the control group and three treatments of 10 ppm, 100 ppm and 250 ppm. An increase in cell number can be observed in the 250 ppm treatment group.

Figure 1C shows the number of cells per area (mm^2) at time=0 and 5 days of exposure in the control condition, as well as the culture treated with 100 ppm nucleotide and the culture treated in the starving condition.

Discussion

Utilizing cells to study additives is a widely used tool in mammalian studies and reduces the cost of developing new biotechnology [13]. Recently, this type of testing has been used in fish as a way to evaluate procedures [14] and to obtain functional information about additives [15] and their nutritional effects [16]. We using SHK-1 cells, in a starving condition for see the effects of nucleotides, in a cellular approach for improve the used of this additive in the industry, at the present time the additive is used but the complete function or mechanism are not complete described in the salmon industry.

We found that nucleotides did not alter cellular viability or promote proliferation in this cell line (Figure 1), we suggested because the culture media have a basal values of nucleotides in the SFB and not need this additives Nucleotides have been described as being good additives in other cellular models [17] and can function as such in fish [18], but we not see basal effect and we proposed a new condition for explorer the physiological effect of this additives in our cells model. We explored the effects of nucleotides on cultures exposed to a starving condition. We simulated a starving condition using a basal medium without serum (Figure 1B), as we did not supplement the medium with fetal bovine serum (FBS). We found that the effects of starving

were reduced when the culture was exposed to 100 ppm of nucleotide (Figure 1C), as this value of nucleotide concentration protects the culture when nutritional conditions are reduced.

Our findings indicate that nucleotide concentrations of 100 ppm are not sufficient to promote cellular division in SHK-1 cells alone. This may be because the serum contained enough concentration of base nucleotides to help the cell culture survive and to significantly reduce the toxic effects in the cellular injury model when the starving condition was generated. This suggests that in these conditions, nucleotides are an additive that functions to protect cells. These results are applicable to the formulation of fish diets, and further studies should focus on the bioavailability of nucleotides and the effect that they could have on aquaculture.

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