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An alternative method for mitotic stimulation in fish cytogenetics

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Abstract. The *in vitro* mitotic stimulation, using *Saccharomyces cerevisae* suspension at glucose-enriched medium, has been widely adopted as a basic methodology for the increasing of mitotic index in fish species, due to its reduced costs, easy performance and good efficacy. However, when used in small, fragile or stressed specimens, it frequently causes a high mortality level. In the present work, the marine fish species *Abudefduf saxatilis*, *Stegastes fuscus*, and *S. pictus* were used to evaluate the efficacy of the pharmaceutical compound Munolan (Allergan Frumtost), an association of bacterial and fungal antigens, as mitotic agent. Comparative analyses among different treatments using *Saccharomyces cerevisae*, Munolan or a combination of both were performed. The best efficacy was obtained by the isolated use of Munolan, coupled with a mortality level decreasing. The results showed the efficiency of the method, encouraging its use for routine cytogenetical studies in fish.

Keywords: Fish cytogenetics, Munolan, Mitotic stimulation, Pomacentridae

Introduction

One of the greatest challenges of fish cytogenetics, especially in the case of marine species, is the obtaining of an adequate number of metaphase plates for the establishment of diploid value and performance of chromosomal bandings. Thus, the *in vivo* stimulation technique by using a glucose-enriched solution of *Saccharomyces cerevisae* (Lee and Elder, 1980), further adapted for fish (Cavallini and Bertollo, 1988), has been incorporated as a routine procedure for chromosome obtaining.

Some authors have reported a successful use in fish cytogenetical studies of horse serum (Ojima and Kurishita, 1980), phytohemaglutinin (Takai and Ojima, 1995), cobalt chloride (Margarido, 1995) or natural infected animals by the ectoparasite *Ichthiophirium multifilis* as mitotic agents.

In the present work, the adoption of the pharmaceutical compound Munolan (Allergan Frumtost), an association of bacterial and fungal antigens, was tested, as an alternative for the use of yeast suspension.

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Materials and methods

The different tests were carried out on damselfish species *Abudefduf saxatilis*, *Stegastes fuscus*, and *Stegastes pictus* (Pomacentridae), all common over rocky and reef areas of the Brazilian Northeastern shore. The specimens used for stimulation experiments presented different body sizes, according to the characteristics of each species (*A. saxatilis*, 10-150 g, *S. fuscus*, 15-40 g, *S. pictus*, 2-8 g). The metaphase quantification was carried out by counting of metaphase plates per slide. Intraperitoneal and intramuscular applications of the antigens were performed in a proportion of 1 ml of solution per 50 g of body weight, over a period from 24 to 54 h prior to chromosomal preparations. The individuals were submitted to four different treatments, (T1) control test, where animals were cytogenetically analyzed without a previous stimulation; (T2) inoculation of glucose-enriched suspension of *Saccharomyces cerevisae* (3 g of fresh yeast + 6 g of dextrose + 25 ml of water, heated for 20 minutes at 37°C), as described by Lee and Elder (1980); (T3) inoculation of Munolan (Allergan Frumtost) solution (76 mg diluted in 2.5 ml of distillate water at room temperature), and (T4) simultaneous inoculation of yeast and Munolan suspensions, a combination of T2 and T3 treatments.

Somatic metaphases were further obtained by direct

(Bertollo *et al.*, 1978) or *in vitro* procedures (Gold *et al.*, 1990), using kidney tissue fragments.

Results

The quantitative analysis of metaphases in the reef species *Abudefduf saxatilis*, *Stegastes fuscus*, and *S. pictus* (Fig. 1a, b, and c) submitted to different treatments of mitotic stimulation (T2, T3, and T4) revealed increasing from 10 to 20 times fold in relation to control treatment (T1) (Table 1).

The raise of stimulation period in the treatment T2, from 24 to 48 h, improved its efficacy (2 times fold). Similar efficacies were observed between periods of 48 h and 54 h. Yet, longer stimulation periods (48 to 54h) using *Saccharomyces cerevisae*, caused a mortality level increasing ; 10% higher than 24 h period and 30% higher than other treatments conditions (T3 and T4).

The treatment T3/24 h, using Munolan, showed to be twice more effective than T2/24 h and had the same efficacy of T2/48 or 54 h. The index obtained by the treatment T4/24 h was nearly half of that obtained by the isolated use of Munolan (T4).

Discussion

Due to fish peculiarities, it is common to obtain a reduced quantity and quality of chromosomal preparations, directly associated to a low mitotic index. This condition is particularly found in marine species (Galetti *et al.*, 2000) or cold-water fishes (Jankun and Rab, 1997). By this way, the mitotic stimulation has been incorporated as a key issue for successful cytogenetical analyses.

Several stimulation methods, as for instance, phytohemaglutinin or horse serum inoculation, natural infection by *Ichthiophirium*, or the use of cobalt chloride, couple some disadvantages such as costs, time/random conditions or health human risks, respectively ; in such case these procedures are less employed. The adoption of yeast suspension is the most used method and has regularly determined the best results for different fish species (Cavallini and Bertollo, 1991). The treatments performed using yeast suspension demonstrated a 10 to 20 times fold increasing of the mitotic index in relation to not stimulated individuals. Longer periods of stimulation T2/48 and 54 h demonstrated to be better than 24 h, suggesting that these should be the periods of maximum immunological response. By using Munolan (T3) this period can be shortened, possibly due to synergic effects of a greater number of antigens. The *in vivo* growth of yeast, after inoculation, sometimes leads to gas production into abdominal cavity, what seems to determine a mortality level up to 30%, if compared to other treatment conditions.

Unexpectedly, a weaker response was observed in the treatment T4 (T2+T3). This fact can be related to in vivo growth of yeast masking and overwhelming the action of Munolan antigens.

The absence of mortality in the samples analyzed under T3 treatment with Munolan compound, associated to a 20 times fold increasing of mitotic index in relation to control treatment reveals an additional advantage for its utilization. Thus, this procedure could be a viable alternative for fish cytogenetics surveys, especially when applied to rare, small, fragile or injured specimens.

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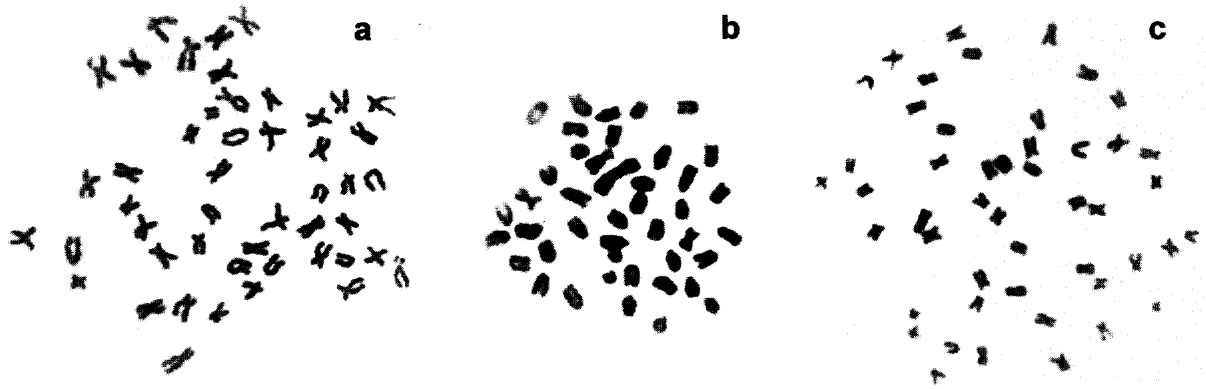

Figure 1. Somatic metaphases obtained by different treatments, (a) *Stegastes fuscus* (T3), (b) *Abudefduf saxatilis* (T2) and (c) *Stegastes fuscus* (T4).

Table 1. Mean frequency of metaphases per slide and efficacy of different treatment for mitotic stimulation in the species *Stegastes pictus*, *S. fuscus* and *Abudefduf saxatilis*.

Treatment	Species	Sex	No	Antigen/time	Metaphase/ slide
T1	*S. pictus*	F	384	Not stimulated-24 h	0
T1	*A. saxatilis*	M	130		3
				Mean	1, 5
				*Efficacy	1, 0
T2	*A. saxatilis*	M	086		8
T2	*A. saxatilis*	F	076	*S. cerevisae*-24 h	14
T2	*A. saxatilis*	F	077		12
T2	*S. fuscus*	F	321		40
				Mean	1, 5
				*Efficacy	12, 3
T2	*S. fuscus*	M	325		43
T2	*S. fuscus*	M	071†	*S. cerevisae*-48 h	—
T2	*S. fuscus*	M	326		30
T2	*S. fuscus*	F	327		30
				Mean	34.3
				*Efficacy	22, 9
T2	*S. fuscus*	M	330		31
T2	*S. pictus*	?	382†		—
T2	*A. saxatilis*	F	323†	*S. cerevisae*-54 h	—
T2	*S. fuscus*	F	331		21
T2	*S. fuscus*	F	333		30
T2	*A. saxatilis*	F	334		54
				Mean	34
				*Efficacy	22, 7
T3	*S. fuscus*	M	387		42
T3	*S. fuscus*	M	079	Munolan-24 h	33
T3	*S. pictus*	M	14051		36
T3	*A. saxatilis*	F	318		21
				Mean	33
				*Efficacy	22
T4	*A. saxatilis*	M	320	*S. cerevisae*+	23
T4	*A. saxatilis*	F	319	Munolan-24 h	12
				Mean	17, 5
				*Efficacy	11, 7

*Efficacy=treatment mean value/control mean value. †dead specimens

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