Influence of rearing temperature changes on thrombocytes aggregation in rainbow trout

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Abstract

Fish, as poikilotherms, are subjected to the influence of the environmental temperature. It has been already reported that fish immune system is influenced by temperature; however, no information is available concerning if temperature has any effect on thrombocytes. Thrombocytes from fish reared at 6°C, 10°C and 20°C were assessed for alteration in their aggregation capacity. Thrombocyte percentages were altered by the water temperature at which the fish was reared, decreasing from 19% (at 10°C) to 13% (at 6°C) and increasing to 24% (at 20°C). However, the aggregation capacity was not significantly compromised by these temperature changes in an individual cell basis, which would suggest a capability of this cell, to maintain this indispensable function at different temperatures.

Key-words:
Trout.
Thrombocytes.
Monoclonal antibody.
Aggregation.

Introduction

Thrombocytes are believed to be the platelets equivalent in fish and aggregation has been described as their principal function in rainbow trout[1,2,3], as well as in other fish[4,5,6]. The employment of monoclonal antibodies (MAb) specific to thrombocytes allowed the isolation of a pure population of these cells, facilitating the studies of their aggregatory potential[4,7,8].

Fish, as poikilotherms, are subjected to the influence of the environmental temperature. Several studies have already demonstrated this effect, for example, on the fish immune system, particularly on helper T-cell function[9]. The capacity of trout leucocytes to produce macrophage-activating factor has been reported to be temperature dependent[10]. Kurata et al.[11] and Kurata and Okamoto[12] has demonstrated that carp neutrophils, which are responsible for spontaneous cytotoxic activity, were able to accommodate their cytotoxic activity according to the temperature. Nevertheless, information verifying whether fish thrombocytes are also affected by changes in the temperature is still lacking.

In this report, we checked whether rearing temperature exerts any effect on the aggregation potential of thrombocytes from rainbow trout.

Materials and Methods

Sixty rainbow trout, weighing approximately 150g were separated in groups of 20 fish, and the fish were kept in 100 l tanks, with running water at a temperature of 10 ± 1°C. Fish were fed daily with commercial feed to apparent satiation on commercial feeding. Fish were fed daily with commercial feed to apparent satiation. After one month, one group was acclimated to a water temperature of 20 ± 1°C, and another group was acclimated to 6 ± 1°C. The last group was kept at 10 ± 1°C as a control. The three groups were kept at the respective temperatures for three weeks and then re-acclimated to 10 ± 1°C. Blood samples were

taken from four fish of each group at the intervals of 0, 21, 35, and 42 days after the beginning of the experiment.

Thrombocyte percentages were analyzed by an Epics Elite flow cytometer (Coulter, USA), following the same procedures described in Kfoury et al.[7]. More than 10,000 cells/ group were counted in this flow cytometry analysis. Statistic analysis was conducted using the chi-square test.

Thrombocytes magnetic separation and aggregation studies: blood sample of each fish was centrifuged at 1800 ´*g* for 5 min at 4°C and the buffy coat was taken, washed in RPMI-1640 (Nissui Pharmaceutical Co., Japan) for three times, and pooled group-wise (6°C, 10°C or 20°C). Peripheral blood leucocytes were obtained by one-step discontinuous gradient separation using a Percoll (Pharmacia, Sweden) density of 1.09 g/ml. About 3 X 10^7 cells, suspended in RPMI supplemented with heat-inactivated fetal bovine serum (FBS; Commonwealth Serum Laboratories, Australia), were pre-incubated with the monoclonal antibody TTL-7D11 (anti-trout thrombocytes; Kfoury et al.[7]) for 45 min at 4°C. After washing, cells were incubated with a 1:5 dilution of magnetic bead-conjugated goat anti-mouse IgG antibody for 20 min at 4°C (Miltenyi Biotec GmbH, Germany). Cells were then separated utilizing a magnetic separation system (Magnetic Cell Separator MACS, Miltenyi Biotec GmbH, Germany) following the procedures described in Kfoury et al.[7].

Thrombocytes aggregation was investigated by using U-46619 thromboxane mimetic (9,11-dideoxy-9α 11α-epoxymethano prostaglandin $F_{2\alpha}$;Sigma, USA), a powerful aggregation inducing drug. The aggregatory effect of U-46619 on the thrombocytes was determined using a method modified from Woodward, Smith and Casilla[3] Five ng of U-46619, dissolved in methyl acetate, was mixed with 3×10^6 cells (suspended in 100 ìl of trout serum) and carefully rotated for 10 min at various temperatures (6°C, 10°C and 20°C), corresponding to the group tested in the temperature shift experiment (i.e.: thrombocytes from fish kept at 6°C were assayed for aggregation potential at 6°C, and so on). Control groups were prepared by adding methyl acetate solution. After that, 100 ìl of 1% paraformaldehyde in 0.1 M phosphate-buffered saline was added in order to fix the aggregated cells. The suspensions were observed with a Thoma's hemocytometer where the free cells were counted. Cells were considered to be aggregated when three or more cells were clumped together. The percentage of aggregated cells for the tests and controls were determined as follows:

$$\text{Aggregated cells (\%)} = \frac{\text{Total cells - Free cells}}{\text{Total cells}} \times 100$$

where "Total cells" is the initial total number of cells, and "Free cells" is the number of cells that did not aggregate. Statistical analysis of the aggregation percentages results were carried out using Chi-square (m´n contingency table) test[13].

Results, Discussion and Conclusions

The percentages of thrombocyte during the course of the experiment are shown in Fig. 1. Their percentages were altered by the water temperature the fish were reared, decreasing from 21% (at 10°C) to 13% (at 6C) and increasing to 24% (at 20°C). These changes proved to be statistically significant. Table 1 shows the percentages of aggregation of thrombocytes from fish kept at 6°C, 10°C and 20°C. An important factor observed is that the difference in the aggregation percentages between the control group (methyl acetate) and the U-46619 added group remained constant throughout the whole experiment period, independently from the temperatures fish were reared.

An increase on the aggregation percentages (in both control and U-46619

Table 1 - Aggregation percentages of thrombocytes from rainbow trout reared at different temperatures

Days of experiment	0	21	35	42
Temperature/ aggregation percentages (parentheses)	10°C U(59%) 51%* M (8%)	20°C U(82%) 55% M(27%)	10°C U(68%) 53% M(15%)	10°C U(60%) 54% M (4%)
	10°C U(58%) 54% M (4%)	10°C U(72%) 55% M(17%)	10°C U(66%) 57% M (9%)	10°C U(65%) 60% M (5%)
	10°C U(60%) 55% M (5%)	6°C U(61%) 57% M (4%)	10°C U(62%) 58% M (4%)	10°C U(63%) 58% M (5%)

*The difference in the aggregation percentages between the control group (methyl acetate added; M) and the U-46619 added group (U) remained constant among the various groups throughout the experiment. The negligible variation observed in this constant was not statistically significant $p < 0.01$ (Chi-square test –m ´n table contingency).

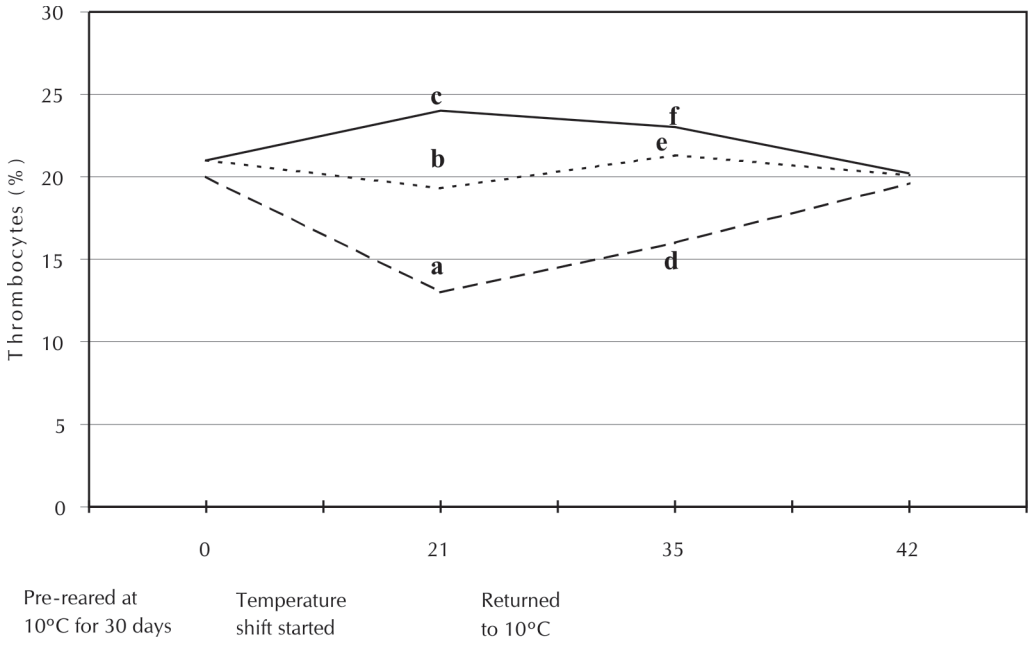

Figure 1 - Thrombocyte percentages of rainbow trout reared at different water temperatures. Percentage values were obtained by FCM analysis. Statistical analysis of the differences among a, b and c, and d, e and f was proved to be significant, $p < 0.01$, Chi-square test. (...) shift to 6°C; (—) kept at 10°C; (___) shift to 20°C

added groups) was noticed in the thrombocytes from fish kept at 20°C. One could speculate that this phenomenon might be due to a hyper-activation of the thrombocyte, however a consistent explanation for this fact is still lacking.

Significant changes on cell percentage have been reported by Kurata and Okamoto[12] where carp head kidney cellular composition (mainly granulocytes and lymphocytes) was altered by the water temperature at which the fish was reared. They have demonstrated that neutrophils increased in number, as well as their cytotoxic activity and binding strength against K-562 cells enhanced at lower temperature, thus concluding that these cells are responsible for carp immune system at lower temperatures, since lymphocytes have their activity severely diminished at these temperatures. Therefore,

the immune system accommodated to preserve its function. Regarding thrombocytes,Casillas and Smith[14] have demonstrated that thrombocytes from rainbow trout (wild and hatchery strains) increased in counts and that blood coagulation becomes more active when fish was submitted to a short-term stress, however, these values returned to normal within 24 hours. In our study, the thrombocyte aggregation capacity (on an individual cell basis) was not significantly compromised by these temperature changes, since the aggregation difference between control and U-46619 added groups remained constant, independently from the temperature fish were kept, which would suggest a capability of these cells to maintain this indispensable function at different temperatures. However, whether the aggregatory capacity (*in vivo*) as a whole is diminished at low temperatures or increased at higher temperatures due to the changes observed in the thrombocyte population still needs to be clarified.

Influência de alterações de temperatura na agregação de trombócitos em truta arco-íris

Resumo

Os peixes, por serem animais pecilotermos, estão sujeitos à influências da temperatura do ambiente. Vários trabalhos já descreveram que o sistema imune é influenciado pela temperatura, contudo não existem informações concernentes à influencia desta sobre os trombócitos. Trombócitos provenientes de trutas arco-íris (*Oncorrhynchus mykiss*) mantidas a 6°C, 10°C e 20°C foram testados quanto à sua capacidade de agregação. A porcentagem de trombócitos sofreu alteração dependendo da temperatura da água em que os animais foram mantidos, diminuindo de 19% (a 10°C), para 13% (a 6°C) e aumentando para 24% (a 20°C). Entretanto, a capacidade de agregação individual de cada célula não foi significativamente afetada por essas mudanças de temperatura, o que sugere a qualidade dessa célula em manter essa função indispensável independente da temperatura.

Palavras-chave:
Truta.
Trombócito
Anticorpo monoclonal.
Agregação.

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