Title: Time and concentration dependency of MacroGard® induced apoptosis

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Abstract

In previous studies an effect of β-glucan on apoptosis in fish was noted and in this investigation we determine the time and concentration dependency of this effect. Primary cell cultures of pronephric carp cells were incubated for 6, 24, 48 h with various concentrations ranging from 0 – 1000 μg/ml of MacroGard® β-glucan. Apoptosis was monitored via acridine orange staining. Results indicate a clear effect of time and concentration on the induction of apoptosis *in vitro*, with only concentration ≥ 500 μg/ml causing significantly higher percentages of apoptotic cells. Apoptosis was detected after 6 h. This concentration dependent effect has to be considered when studying apoptosis in relation to immunostimulation.

Keywords

Apoptosis, beta-glucan, carp, concentration/time dependency

Introduction

A cost effective approach to disease prevention in farming of aquatic and terrestrial animals is the administration of substances that enhance the immune system. These substances, known as immunomodulators or immunostimulants, are utilized to increase general protection against disease or to overcome the immunosuppressive effects of stress [1]. Generally substances that have immunostimulatory functions act as pathogen associated molecular patterns (PAMPs) and induce an immune response by interaction with pattern recognition receptors (PRRs). One such PAMP is β-glucan, a carbohydrate that is an essential cell wall component of fungi, bacteria, algae, oats and barley [2]. β-Glucan is an ideal target for studies on disease prevention since this substance stimulates various immune responses and enhances protection against viral and bacterial pathogens [3]. It also occurs naturally in the environment and therefore raises less concern in regards to adverse impacts on the environment and human health [4].

In humans β-glucans display various medicinal properties for example, decreases in cholesterol levels, enhancement of wound healing, and inhibition of cancer cell growth [5]. In mammals although various receptors e.g. complement receptor C3, dectin-1 and TLR1/6 have been described [6], dectin 1 is considered as major β-glucan receptor [7]. Differential responses are elicited when β-glucan binds to dectin-1 alone or together with other receptors such as TLR 2 [8]. However, dectin-1 could not be identified in fish and it has been suggested that β-glucan is detected by multiple pattern recognition receptors including toll like receptors [9]. In both mammals and fish, β-glucan recognition results in the activation of macrophages, which induces phagocytosis, leukocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species, as well as the enhancement of complement activity [9-17].

However, the immunostimulating effects of β-glucan have been shown to be dependent on dose, duration of administration, environmental temperature and the species. For example, no effect of β-glucan on stress related parameters were observed in channel catfish (*I. punctatus*) [18], whilst Jeney

and colleagues [1] observed stress reducing effects of dietary β-glucan in rainbow trout (*O. mykiss*). This effect observed by Jeney et al. was however dose dependent and only occurred at 0.1 % β-glucan, whilst at 2 % β-glucan administered in feed led to a suppressed immune response which seemed to render the animals more susceptible to an infection. In fish, studies have shown that β-glucan dosage in the feed affects the respiratory burst activity of macrophages leading to differences in time and height of the peak respiratory burst activity [19]. These possible adverse dose effects of β-glucan have also been noted in crustaceans. For example, Hauton and Smith [20] noted that in lobster an increase in β-glucan concentration decreased the viability of granulocytes.

The possible mechanisms of the dose dependent effects of β-glucan particularly the reduction of immunostimulation at high doses have not been ascertained. However, in a recent detailed study by Kepka and coworkers [21] zymosan, a β-1,3-glucan from yeast, induced apoptosis *in vitro* and *in vivo*, which was linked to the production of ROS in carp leukocytes. Unfortunately the dose dependency of this phenomenon was not ascertained. The ability of β-glucan to induce apoptosis-related genes has previously been noted in fish by Miest et al. [22]. In fact, the β-glucan induction of apoptosis can be beneficial as β-glucan can also affect programmed cell death in human cancer cells. For example, Kim et al. [23] have shown that bacteria-derived β-glucan can induce apoptosis and that this form of cell death is involved in the tumouricidal effects of β-glucan [24, 25].

In this study the dose effect of β-glucan, in the form of MacroGard®, was investigated to ascertain its relationship with apoptosis in the pronephric leucocytes of fish. The pronephros was chosen as target organ as it fulfills important immune functions such as haematopoiesis, phagocytosis and antigen processing in the fish and Verburg-van Kemenade and colleagues [30] identified lymphocytes, neutrophilic and basophilic granulocytes and macrophages in suspensions of isolated pronephric cells. This heterogenous cell suspension hence allows us to study the effect of MacroGard® on a wide variety of immune cells.

The concentrations of MacroGard® utilised corresponded to concentrations used in other investigations involving different experimental animals for example, 1 – 200 µg/ml for mouse

macrophages [26], 100 – 800 µg/ml for porcine leucocytes [27], 1 µg/ml for macrophages of rainbow trout (*Oncorhynchus mykiss*) [28], and 0.5 – 500 µg/ml for the phagocytes of turbot (*Psetta maxima*), gilthead sea bream (*Sparus aurata*) [29] and 500 µg/ml in carp (*Cyprinus carpio*) [21].

Materials & Methods

Common carp (*Cyprinus carpio*) were obtained from Fair Fisheries, Shropshire, England and reared in black 1 m x 0.5 m tanks with 225 litres of 15 °C dechlorinated water at pH 7. The water in each tank was circulated and cleaned by a temperature regulating biological pond filter (Eheim). Fish were fed daily with commercial dry pelleted food (Tetra Pond feed) and kept on a 12 hour light/dark cycle. The head - tail length of the fish ranged from 7.9 to 18.1 cm (mean length 10.6 cm) and weight ranged from 8.2 to 89.7 g (mean weight 36.3 g). For organ sampling fish were removed from the tank by netting and sacrificed with a lethal dose (~ 0.2 %) of 2-Phenoxyethanol (Sigma Aldrich, P1126) in aquarium water.

A pronephric cell suspension was prepared using a modification of the procedure described by Verburg-van Kemenade and coworkers [30]. In brief, the organ was gently disrupted through a sterile cell strainer with 100 µm pore diameter (BD Falcon Cell strainer, Scientific Laboratory Supply, 352360) in 1 ml of modified RPMI medium. The modified cell culture medium (hereafter referred to as RPMI+) consisted of RPMI with 0.3 g/L L-glutamine (Sigma Aldrich, R7388) with 0.5 % sterile water, 0.05 % pooled carp serum, 0.05 mM β-mercaptoethanol (Sigma Aldrich, M-3148), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Sigma Aldrich, P4458). The viability of the cell population was ascertained with trypan blue staining and only cell suspensions with a viability of at least 95 % were used. The concentration of the cell suspensions was adjusted to 1 x 10^7 cells/ml with RPMI+ medium. Cells were set up in 96 well plates (Sarstedt, UK) with 100 µl per well.

MacroGard® was provided by Biorigin (Brazil), according to the certificate of analysis the MacroGard® batch (batch 250813) consisted of 67.8 % carbohydrates, 5.1 % protein (dry matter),

14.2 % lipids, 6.5 % ash and 4.6 % moisture. The microbiological analysis was negative for *Salmonella* sp., *Escherichia coli* and Coliforms (analysed by Biotech Pharmacon).

A stock solution of MacroGard® in autoclaved deionized water (sH_2O) was sonicated (2 x 30 s at power 6, Sonics, vibra-cell). Sterility was ensured by pasteurisation of the solution in a water bath at 80 ºC for 20 min and left at room temperature to cool down. Concentrations of MacroGard® were prepared from the stock solution in sH_2O as required. Following concentrations were used: 1, 50, 100, 250, 500, and 1000 µg/ml, which correspond to 0.6, 30, 60, 150, 300 and 600 µg/ml β-glucan. In addition, three controls were set up: an untreated control (i.e. no additives), a control with H_2O as additive, and a positive UV exposed (324 J/m^2) control as described in [31]. Cells where then incubated for 6, 24 or 48 h at 20°C, and apoptosis visualized as described in [22] using acridine orange.

Prior to statistical analysis the data were arcsin transformed to meet the assumption of normal distribution. The data were analysed for the influence of exposure time (i.e. 6, 24, and 48 h) and the various treatments (i.e. controls and MacroGard® concentrations) with 2-way repeated ANOVA. For the analysis of the distinct effects the treatments had on apoptosis a 1-way ANOVA was performed for the individual time points with a subsequent Turkey's post-hoc test. Analyses were performed using Minitab Release 14 and GraphPad Prism 4.

Results

The concentration of MacroGard® ($F = 12.39$, $p < 0.0001$) and the exposure time ($F = 20.73$, $p < 0.0001$) had a significant effect on apoptosis levels. Additionally an interaction was found between the main effects ($F = 1.98$, $p < 0.05$). As this global test indicated that the effect of dose varied with time, a series of one-way ANOVAS and post-hoc tests was conducted to examine where these differences lie (Figure 1).

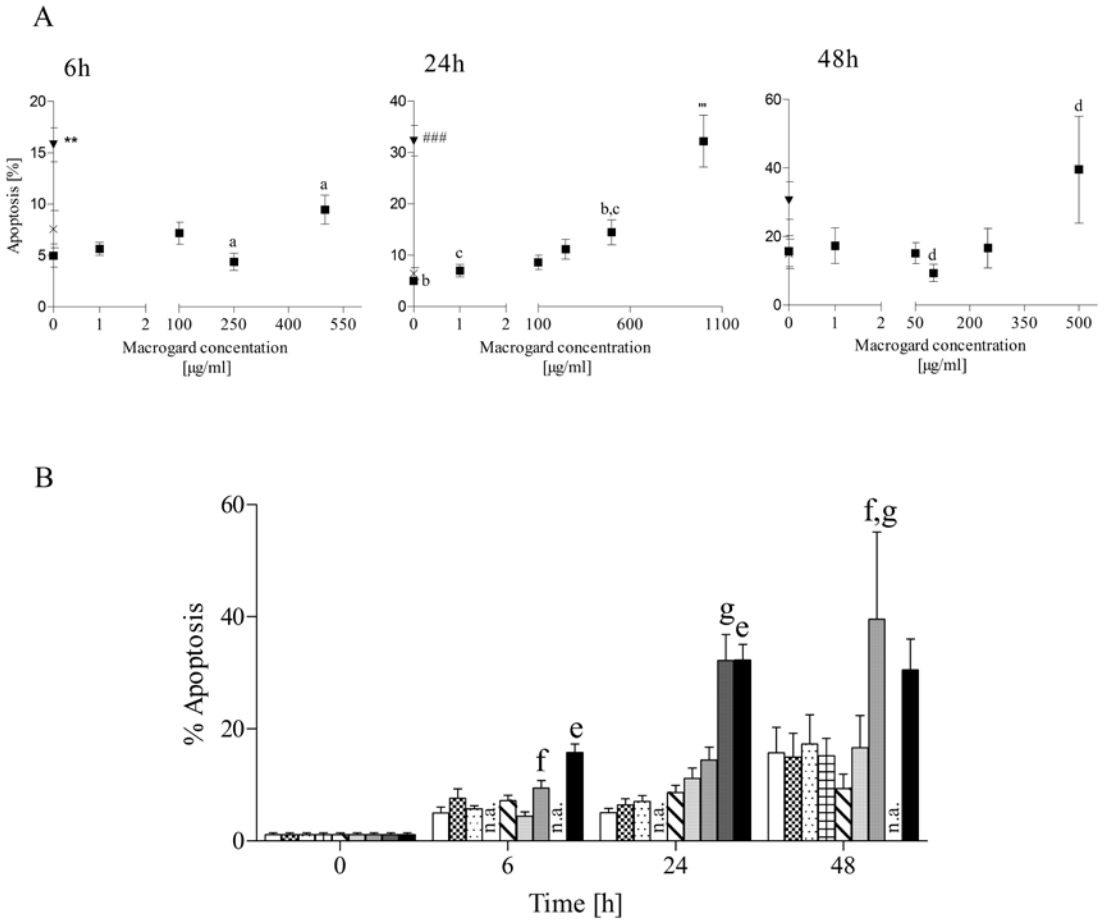

Figure 1: Time and concentration dependency of MacroGard® influence on apoptosis

Percentage of apoptotic cells in the cell suspension was analysed with acridine orange. Cell suspensions were treated with different concentrations of MacroGard® and incubated over 3 different time periods. Significance was defined as $p \leq 0.05$. Symbols for significant differences: * = significantly different to all other samples with ** = $p \leq 0.01$; ### = significantly different to all other samples except 1000 µg/ml with $p \leq 0.001$; ''' = significantly different to all other samples except UV with $p \leq 0.001$, same letters indicate differences between samples (with a, c, d, f = $p \leq 0.05$, b = $p \leq 0.01$, f, g = $p \leq 0.001$). n.a. = not analysed. Data are shown as mean ± SEM with n = 8. A) Concentration dependency of MacroGard® effects at different time points. X = water control, ▼ = UV control, n = 6 – 8. B). Time dependency of various MacroGard® concentrations. ■ = UV, ▩ = 1000 µg/ml, ▩ = 500 µg/ml, ▩ = 250 µg/ml, ▩ = 100 µg/ml, ▩ = 50µg/ml, ▭ = 1 µg/ml, ▩ = water, ▭ = 0 µg/ml.

At all the time points there was no significant difference detected in apoptosis levels of the two negative controls (i.e. untreated and with added water). By running a positive control (i.e. UV exposure) alongside the samples it was established that apoptosis could be induced in the tested cell population. This UV exposure caused higher percentages of apoptosis positive cells after 6 hours (15.8 ± 1.54 %) and 24 hours (32.3 ± 2.8 %) when compared to the non-treated control (both $p \leq 0.001$). When cells were exposed to MacroGard® for 6 hours none of the tested concentrations (1 – 500 μg/ml) induced apoptosis in the pronephric carp leucocytes. Most effects were observed after 24 h of MacroGard® exposure. At this time point 500 μg/ml induced significantly higher apoptosis in the cell culture (i.e. 14.5 ± 2.4 %) compared to the non-treated control (5.1 ± 0.7 % apoptosis) ($p \leq 0.01$) and the 1 μg/ml MacroGard® concentration (7.0 ± 1.1 % apoptosis) ($p \leq 0.05$ and 0.001 respectively).

At 24 h, a concentration of 1000 μg/ml of MacroGard®, included in the experimental design to test if concentrations > 500 μg/ml induced significantly higher apoptosis (32.2 ± 5.0 %, $p \leq 0.001$), induced similar to apoptosis levels as the positive UV control. Because of the relatively high levels of apoptosis therefore this concentration was not tested at the other time points utilised. We therefore included an additional concentration of 50 μg/ml at the 48 h time point. As at 24 h 500 μg/ml MacroGard® induced significantly higher apoptosis levels (39.56 ± 15.57 %, $p \leq 0.05$ compared to 100 μg/ml). Additionally, there was a significant difference between the apoptosis levels caused by 500 μg/ml at 48 h and 24 h and 6 h (both $p \leq 0.001$). None of the other tested concentrations were significantly different.

Discussion

The 2010 report by the Food and Agriculture Organisation of the United Nations on fisheries and aquaculture [32] stated that aquaculture will overtake capture fisheries as the primary source of food fish in the future. However the growth of this food-producing sector is impaired by disease outbreaks, which both reduce productivity in fish farms and pose a problem for biosecurity. Immunostimulation is an important tool in aquaculture to increase resistance to pathogens [3] especially since substances, such as β-glucan, occur naturally in the environment and are thus less likely to raise concerns about residues in food fish and the environment [4]. Previous studies on β-glucan have highlighted a time and dose dependency of the effects [1, 33-39].

However for many substances used as immunostimulants, including LPS and β-glucan, effectiveness is dose dependent, such that high concentrations often lead to adverse effects, including immunosuppression [19, 38, 40, 41]. As a result the resistance of the animals to pathogens is not enhanced or is even decreased at high concentrations, and hence it is important for both feed manufacturers and fish farmers that the dietary dose of the immunomodulating substance utilized does not induce such negative effects. In the present study, as well as in several other studies, it has been demonstrated that the effects of β-glucan are dose dependent [20, 29, 35]. For instance the cell viability in lobster granulocytes decreased with an increase from 50 to 250 μg/ml in MacroGard® concentration [42]. The respiratory burst activity in response to MacroGard® exposure is also dependent on the applied concentration as demonstrated by Castro and coworkers [29]. In their report concentrations ranging from 0.5 to 5 μg/ml had no effect on the production of ROS while concentrations of 7.5 to 500 μg/ml significantly induced the respiratory burst. In addition, the authors investigated the stimulatory effects of MacroGard® and found that low concentrations (i.e. 1 – 2.5 μg/ml) led to higher ROS production after secondary stimulation with another ROS inducing agent compared to cell cultures that were not pre-treated with MacroGard®. In the same study higher concentrations of MacroGard® (i.e. 10 – 500 μg/ml) inhibited the ROS response to the secondary stimulation, which was interpreted by the authors as a sign of exhaustion of the cells. However in regard to the findings described in this manuscript it is possible that the lower ROS production could

be due to the onset of apoptosis and thus lower cell numbers in the culture. Reactive oxygen species are known for their cytotoxicity in fish [43, 44] and hence it is possible that the higher β-glucan concentrations induced ROS, causing apoptosis which then results in lower respiratory burst activity due to the reduced cell numbers.

Nonetheless, even though reports have shown that β-glucan has dose-dependent immunostimulating and immunosuppressing effects, up to now the mechanisms behind these differential effects have not been elucidated. It is interesting however that Kepka et al. [21] showed that *in vitro* 500 µg/ml zymosan and *in vivo* 40 µg zymosan/g body weight can induce apoptosis in pronephric granulocytes and macrophages (*in vitro*), as well as peritoneal leukocytes (*in vivo*) in carp.

The findings of our study and the work conducted by Kepka et al. [21] suggest that some of the immunosuppression noted may be associated with the induction of apoptosis in immune cells, which are exposed to particular high doses of β-glucan. In our studies the pro-apoptotic effect was noted to be time and dose dependent with only concentrations of ≥ 500 µg/ml causing apoptosis in pronephric leucocytes *in vitro*. This is perhaps not surprising as most substances are toxic above a certain threshold [45]. This concentration dependency is therefore an important factor to be considered during dietary administration of an immunostimulant. In aquaculture situations it is therefore important to administer β-glucan at concentrations which are high enough to stimulate the immune response, but also low enough to avoid any possible adverse effects due to high doses. This supports our previous observations [10, 11, 13, 16, 22] in which we showed that MacroGard® administered at the producer recommended (10 - 15 mg/kg bodyweight) dose does not induce apoptosis in pronephric cells but stimulates the immune response.

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