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A Rapid, Direct Assay to Measure Degranulation of Primary Granules in Neutrophils from Kidney of Fathead Minnow (Pimephales Promelas Rafinesque, 1820)

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Keywords
neutrophils, fish, degranulation assay, fathead minnow, Veterinary Microbiology & Preventive Medicine

Comments
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Abstract

A direct, rapid, quantitative colorimetric assay to determine neutrophil primary granule degranulation was adapted for use with fathead minnow kidney neutrophils. The assay measures the exocytosis of myeloperoxidase (MPO) using 3,3′,5,5′-tetramethylbenzidine as a substrate. The assay was validated by comparing the total myeloperoxidase content of neutrophil populations obtained from adult cattle, as a known positive, and fish; evaluating the effects of calcium ionophore (CaI), phorbol myristate acetate (PMA), aqueous solution of β-glucan (MGAQ) and zymosan (Z) with and without cytochalasin B (cyto B) as stimulants of degranulation; determining the kinetics of primary granule exocytosis and detecting changes in degranulation when fish were exposed to stress and anaesthesia with MS-222. The MPO assay detected MPO activity in fathead minnow neutrophils that correlated to neutrophil numbers, confirmed that degranulation was increased when CaI was used compared to other stimulants, determined degranulation peak at 60 min and confirmed decreased degranulation after exposure to handling and crowding stress, with and without MS-222. Therefore, the MPO assay is capable of detecting important differences that may occur in degranulation of fathead minnow kidney neutrophil primary granules and in total neutrophil myeloperoxidase content.

Keywords: Neutrophils; Fish; Degranulation assay; Fathead minnow

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1. Introduction

Neutrophils are an important component of host defence against many bacterial, viral and fungal infections, and the evaluation of neutrophil function is valuable for assessment of the health status of individuals and animal populations [1]. Neutrophil populations have been purified to study cell function and to investigate the pathogenesis of many human and some animal diseases [1–4].

Fish leucocytes have been classified using criteria that primarily apply to mammalian counterparts [5] and several studies have suggested that many fish leucocytes show morphological resemblance and functional similarities with mammalian cells [6–8]. Most studies document that fish neutrophils have very similar histochemical staining properties to mammalian neutrophils, and can be distinguished by the presence of myeloperoxidase (MPO) in their cytoplasmic granules [6,9,10]. Neutrophils are found in fish kidney, spleen and blood, and are commonly increased in inflammatory lesions [9,11]. There is evidence for phagocytic, chemotactic and bactericidal functions in fish neutrophils and an intense respiratory burst [11], and these parameters can be used for health status assessment [12].

Different granule types within the mammalian neutrophils contain a variety of antimicrobial substances that are released into phagosomes or to the cell exterior during degranulation [13]. Degranulation of neutrophil primary granules has been measured using indirect methods such as iodination assay or exocytosis assay [14]. Measuring exocytosis of myeloperoxidase from primary neutrophil granules in vitro is a direct, rapid and quantitative method to assess the degranulation process in neutrophils [15]. The assay based on MPO–H_2O_2 oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) is considered the least toxic of the peroxidase sensitive substrates [15–17]. The assay was adapted for use with fathead minnow (*Pimephales promelas* Rafinesque, 1820) kidney neutrophils using a microtitre plate method previously described for bovine and human neutrophils [15,16].

The fathead minnow is a scientifically and ecologically important fish species [18–20] extensively used in toxicology research [21,22], and a significant forage species for larger predator and game fish [23]. Techniques for obtaining relatively pure neutrophil populations have been previously reported for this species [10], and the characterisation of leucocyte morphology and cytochemical staining demonstrated the presence of myeloperoxidase in fathead minnow neutrophil granules [10].

It recently has been shown that anaesthesia in fish using tricaine methanesulphonate (MS-222, Finquel) does not prevent increase in plasma cortisol levels during handling and crowding stress [24]. Increased plasma cortisol levels can indicate stress conditions in fish, and the biological effects of cortisol include suppression of immune system responses [25]. Therefore, handling and crowding stress, with and without anaesthesia, was used to reduce neutrophil MPO release and demonstrate the ability of the MPO assay to measure the decrease in primary granule exocytosis. In this study, a rapid, direct MPO assay to measure degranulation of primary granules of neutrophils from the fathead minnow kidney is described. Evaluation of neutrophil function in this animal model will provide valuable information and become a useful tool in ecotoxicology research [26].

2. Materials and methods

2.1. Fish

Adult fathead minnows with an average weight of 3 g were maintained in the Department of Natural Resource Ecology and Management, Iowa State University, Ames, Iowa, USA. Fish were held in a 300–1000 L tank recirculation system supplied with dechlorinated tap water at 20 °C and fed daily with dried flake food (Aquatox®, Ziegler Bros Inc, PA, USA). Fathead minnows were cared for in accordance with approved Iowa State University animal care guidelines.

2.2. Neutrophil separation

Fathead minnows were caught in a net and immediately killed by immersion in 1 g L^{-1} tricaine methanesulphonate (MS-222, Finquel, Argent Chemical Laboratories) solution. Kidney tissue from 10 individual fish was aseptically collected and neutrophils were separated using a previously described technique [10]. Briefly, kidneys were pooled in Hank's balanced salt solution without Ca, Mg and phenol red ($HBSS^{CMF}$, Mediatech-CellGro, AK, USA), homogenised in a 15 mL tissue grinder (Wheaton, USA) and pelleted for 15 min at $250\times g$. The cell pellet was resuspended in $HBSS^{CMF}$ and gently placed over separation medium with a specific gravity of 1.078 g mL^{-1} (Lymphocyte separation medium 1078, Mediatech-CellGro, AK, USA). Gradients were centrifuged for 30 min at $400\times g$, the cells at the interface were removed, washed, resuspended in $HBSS^{CMF}$ and total leukocyte counts and viability (trypan blue dye exclusion) were determined using a Neubauer-ruled haemocytometer [10,27,28]. Cell suspensions were adjusted to a standard concentration of 2.5×10^7 cells mL^{-1} and the neutrophil ratio was determined by differential leukocyte counts on Hemacolor (Harleco, EM Science, NJ, USA) stained cytospin preparations of cell isolates. Identification and morphological characterisation of leucocytes were done using suggested criteria [5,6,10].

2.3. Reagents

Working solutions of 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, Sigma; 2.5 mM in water) and hydrogen peroxide (H_2O_2, 5 mM in water) were prepared immediately before use. Sulphuric acid (H_2SO_4, Fisher; 2 M) was used as a reaction stop solution. The detergent cetyltrimethylammonium bromide (CTAB, Sigma; 0.02% in water) was used as a lysing agent for determining total myeloperoxidase content of neutrophils. Stock solutions of Cytochalasin B (cyto B, Sigma; 1 mg mL^{-1}), phorbol myristate acetate (PMA, Sigma; 1 mg mL^{-1}) and calcium ionophore A23187 (CaI, Sigma; 1 mg mL^{-1}) were prepared in dimethyl sulphoxide (DMSO, Sigma), and stored at −70 °C. A stock solution of zymosan (Z, 10 mg mL^{-1}) was prepared in Hank's balanced salt solution with Ca, Mg and without phenol red (HBSS, Mediatech-CellGro, AK, USA), and stored at −70 °C [14]. A stock solution of MacroGard AquaSol (MGAQ, 1% solution of water soluble β-1,3-glucan from baker's yeast, courtesy of Biotec Pharmacon ASA, Tromso, Norway) was aliquoted and kept at 4 °C until use. Aliquots of the reagents were diluted in HBSS for each assay. Preliminary titrations were used to determine optimal reagent concentrations. The final concentrations of reagents used in the assay after addition of HBSS and resuspended neutrophils were: cyto B 5 μg mL^{-1}, CaI 5 μg mL^{-1}, PMA 1 μg mL^{-1}, Z 200 μg mL^{-1} and MGAQ 33 μg mL^{-1}.

2.4. Assay method

Release of myeloperoxidase from neutrophils in response to CaI, PMA, Z and MGAQ with and without cyto B was determined using 96-well flat bottom microtitre plates (Linbro Titertek, USA). Test wells received 75 μL of cyto B or HBSS (if release without cyto B was measured) and 50 μL of stimulants. Control (background) wells received 125 μL of HBSS. Total MPO content wells received 125 μL of CTAB solution. Total MPO and background values for each trial were determined concurrently with neutrophils exposed to stimuli. All samples were tested in duplicate wells. Plates containing reagents were pre-warmed to 30 °C, 25 μL of cell suspension containing 2.5×10^7 cells mL^{-1} was added to each well and incubated at 30 °C for 20 min. To study the kinetics of MPO release, CaI and PMA (with or without cyto B) stimulated neutrophils were incubated for times varying from 0 to 60 min. After incubation, 50 μL of TMB was added, followed immediately with 50 μL of H_2O_2. The colour change reaction was allowed to proceed for 2 min, and 50 μL of 2 M sulphuric acid was added to stop the reaction. Test plates were centrifuged at $600\times g$ for 15 min, 200 μL of supernatant from each well was transferred to another plate and optical density (OD) in

each well was determined at 405 nM using a microtitre plate spectrophotometer (V-Max, Molecular Devices, USA) with SOFTmax PRO 4.0 software.

The total myeloperoxidase content was calculated from the OD of lysed neutrophils using a standard curve. Standard suspensions of bovine neutrophils (5×10^7 cells mL^{-1}) with phenylmethylsulfonyl fluoride (PMSF, Sigma; 1 mM in absolute ethanol) were prepared and stored at −70 °C. Two-fold serial dilutions of a standard neutrophil suspension were lysed with CTAB. The OD due to the MPO content of these known concentrations of neutrophils was compared to OD values with serial dilutions of commercially available MPO (EC 1.11.1.7, Sigma), and a standard curve was calculated. Bovine neutrophil suspension with total MPO content of 2.84 units per 10^6 cells was used as an assay standard. One unit of commercially available MPO (Sigma, approximately 0.02 mg protein) produces an increase of adsorbance of 1.0 per min at 470 nm, pH 7.0 and 25 °C, using guaiacol as substrate. Total MPO content for fathead minnow kidney neutrophil suspensions was normalised using the following correction formula:

$$\mathrm{NU}=\frac{(\mathrm{NCW}\times U)}{(\%N\times\mathrm{NCW})/100}$$

where NU is the normalised MPO unit value, NCW is the number of cells per well, U is the measured MPO unit value and $\%N$ is the percent of neutrophils in cell suspension.

The percent release of MPO was calculated using the following formula:

$$\%\ \text{release}=\frac{\left(\mathrm{OD}_{\text{stimulated}}-\mathrm{OD}_{\text{background}}\right)}{\left(\mathrm{OD}_{\text{lysed}}-\mathrm{OD}_{\text{background}}\right)}\times100$$

2.5. Anaesthesia and myeloperoxidase exocytosis

To determine ability of the assay to measure decreases in neutrophil activity, fish were exposed to handling and crowding stress with and without anaesthesia. A total of 360 fish were acclimatised for 2 weeks in a stock tank and used in the experiment. Fish were fed daily and water quality parameters were monitored two times per week for the duration of the acclimation and experiment: water temperature was 20±1 °C, pH was 8.0±0.2, dissolved O_2 was 7±1 mg L^{-1}, total ammonia nitrogen was <1.0 mg L^{-1} and total nitrite nitrogen was below the detection limit (HACH spectrophotometer 2000NR). No mortalities were recorded 2 weeks prior and during the experiment.

At the beginning of the experiment (day 0), 252 fish were quickly netted from the stock tank and randomly divided into the following groups: stressed anaesthetised group (SA, 108 fish), stressed group (S, 108 fish) and control group (36 fish). The SA group was immediately transferred to a 20 L bucket with 10 L tank water buffered with sodium bicarbonate to pH of 8.0, constant aeration, and 75 mg L^{-1} of MS-222. A third degree of anaesthesia was achieved within 3 min from transfer (third degree or surgical anaesthesia: total loss of sensitivity, equilibrium and responsiveness to external stimuli as well as decrease in respiration rate) [29]. The third degree of anaesthesia was maintained for 20 min, and fish were moved to a 1000 L recirculation tank supplied with the same water as the stock tank, and within 5 min, 100% of the fish recovered. Fish from the S group were immediately transferred to a 20 L bucket filled with 10 L of tank water prepared as described above, but without anaesthetic. This procedure provided similar (approximately 30 g of fish per L) handling and crowding conditions compared to fish from the SA group. After 20 min of crowding, S group fish were placed in the same tank as the SA group, but separated with a net that prevented the mixing of fish. Fish from the control group were immediately killed with an overdose of MS-222 (1 g L^{-1}), randomly divided into six samples of six fish each and assayed for neutrophil function as day 0. Six samples (randomly selected six fish/sample) from the SA group, six samples from the

S group and six samples from the control group (remaining 108 fish from stock tank) were assayed for neutrophil function on days 1, 3 and 7 after treatment. The mean % of neutrophil MPO release (determined as described above) from the control group was used as 100% of neutrophil degranulation activity for each day. Neutrophil degranulation activity for the SA and S groups was compared to the activity for the control group using the following formula:

$$\%\ \text{of control group neutrophil degranulation activity} = (\%\ \text{MPO release of SA or S} / \%\ \text{MPO release of control}) \times 100.$$

2.6. Statistical analysis

Data are presented as means ± standard error of the mean (SEM) unless otherwise indicated. The differences in total MPO content of bovine and fish neutrophils and kinetics of degranulation were examined by ANOVA and regression plots (SAS Institute, 2001). The differences in MPO release due to the various stimulants and inhibition of MPO release due to anaesthesia exposure were examined using ANOVA followed by Student's *t*-test (GraphPad Prism 3.00, 1999). $P < 0.05$ was considered significant.

3. Results

3.1. Myeloperoxidase activity in neutrophils

Mean neutrophil purity from the kidney cell suspensions was 70.6 ± 7.6%, and mean neutrophil purity in the spleen cell suspension was 3.7 ± 2%. The remaining cells were lymphocytes and thrombocytes, with less then 1% monocytes/macrophages.

The myeloperoxidase content measured in two-fold dilutions of CTAB lysed cells was considered as 100% release (total MPO release) (Figs. 1 and 2). The MPO content measured in lysed uncorrected fathead

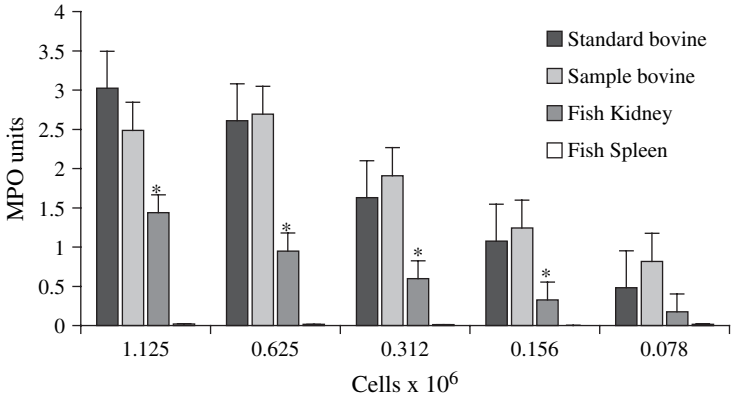

Fig. 1. Total myeloperoxidase content in two-fold dilutions of different cell suspensions. MPO unit values are calculated from absorbance at 405 nm through use of a standard curve [16] without normalisation of kidney and spleen cell suspensions for neutrophil content. Standard bovine neutrophil suspension (dark grey bar) has 98% neutrophils and <1% eosinophils. Sample bovine neutrophil suspensions (light grey bar) have 98 ± 2% neutrophils and 1–3% eosinophils. Kidney cell suspensions (grey bar) have 70 ± 8% neutrophils and have significantly different (*$P < 0.05$) total MPO content from bovine neutrophils at all dilution points except the highest. Spleen suspensions (open bar) have less than 5% neutrophils and less than 0.02 MPO units per 10^6 cells at all dilution points. Data are presented as means ± SEM ($n = 6$).

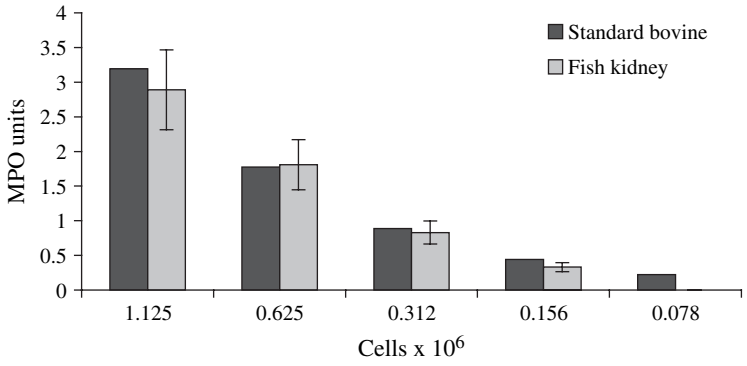

Fig. 2. Total myeloperoxidase content in fathead minnow neutrophils. Kidney cell suspensions (light grey bar) have been normalised for a number of neutrophils, MPO unit values calculated from absorbance at 405 nm and compared with standard bovine neutrophil suspension (dark grey bar). There is a significant decrease in MPO activity with decrease in cell numbers ($R^2=0.948$). There is no significant difference in the activity of normalised kidney cell suspension and bovine standard neutrophils at all dilution points ($P>0.4$). Data are presented as the mean±standard deviation ($n=35$).

minnow kidney cell suspensions was approximately 50% of the content of a standard suspension of bovine neutrophils and sampled bovine neutrophils (1.28, 2.68 and 2.21 MPO units per 10^6 cells, respectively) (Fig. 1). There was a significant difference in MPO content of kidney cell suspensions and bovine neutrophils ($P<0.05$) at all dilution points except for 0.078×10^6 cells. Fathead minnow spleen cell suspensions demonstrated very little MPO content (less then 0.02 MPO units per 10^6 cells). Increasing the cell dilution correlated with decreasing MPO content to undetectable concentrations (Fig. 1). In kidney cell suspensions that were normalised for neutrophils, total MPO content ranged from 2.24 to 2.90 MPO units per 10^6 neutrophils (mean=2.57, SD=0.33, $n=35$) and were not significantly different ($P>0.4$) from standard bovine neutrophils at any dilution point (Fig. 2). Two-fold dilutions of the normalised kidney cell, standard bovine neutrophil, sample bovine neutrophil, and uncorrected kidney cell suspensions showed a significant decrease in MPO content with a decrease in cell numbers ($R^2=0.948$), confirming the ability of the assay to measure differences in total MPO content (Figs. 1 and 2) that correlated to neutrophil numbers.

3.2. Exocytosis of primary granules

Exocytosis of MPO from fathead minnow kidney neutrophils exposed to CaI, PMA, Z and MGAQ is shown in Fig. 3. The effect of each stimulant was measured with and without physiologic levels of extracellular Ca^{2+} and with and without 5 μg mL^{-1} cyto B. In media devoid of Ca^{2+}, MPO release due to stimulants with and without cyto B was <5% (not shown). In medium containing Ca^{2+} but no cyto B, the addition of CaI (47.7% release), MGAQ (22.9%) and Z (9.4%) caused a significant increase in percent of MPO release compared to control ($P<0.01$). PMA did not cause a significant increase in percent of MPO release when applied without cyto B, regardless of the presence of Ca^{2+}. In medium with Ca^{2+}, cyto B alone caused <5% MPO exocytosis (not shown).

A significant increase in percent of MPO release compared to cyto B alone ($P<0.05$) was seen with the addition of CaI (55.9%), PMA (44.7%) and MGAQ (17%) (Fig. 3). The presence of cyto B caused a significant difference in CaI, PMA, and zymosan induced MPO release ($P<0.05$). Cyto B was only necessary for induction when PMA was used (Fig. 3). Cyto B inhibited zymosan-induced exocytosis and had no significant effect on MGAQ-induced exocytosis (Fig. 3).

The kinetic study of MPO release (Fig. 4.) demonstrated that neutrophil primary granule exocytosis in response to stimulation by either CaI or PMA, and in the presence or absence of cyto B, can be detected at

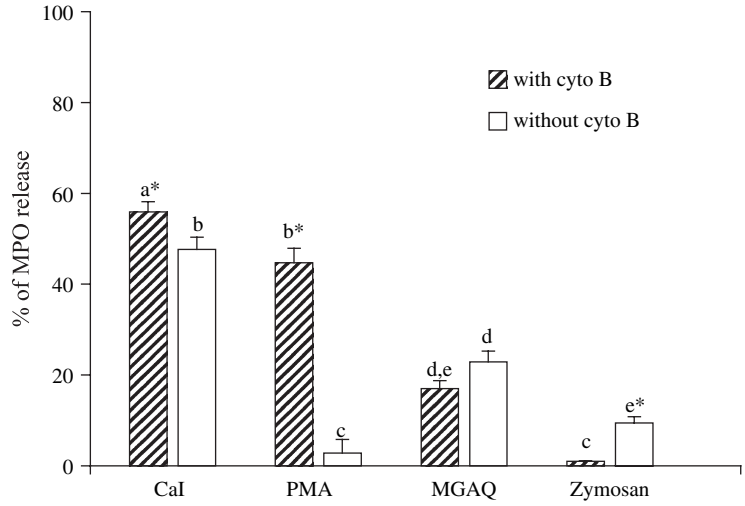

Fig. 3. The effect of various stimulants on fathead minnow kidney neutrophil myeloperoxidase (MPO) exocytosis in the presence or absence of 5 μg mL^{-1} cytochalasin B (cyto B). The striped bars indicate the presence of cyto B. The concentrations of the stimulants are: 5 μg mL^{-1} calcium ionophore (CaI), 5 μg mL^{-1} cyto B, 1 μg mL^{-1} phorbol myristate acetate (PMA), 33 μg mL^{-1} MacroGard AquaSol water soluble β-1,3-glucan (MGAQ), 200 μg mL^{-1} zymosan (Z). Neutrophils pooled from 10 adult fathead minnows were incubated with the stimulants at 30 °C for 20 min. Data are presented as the mean ± SEM ($n=6$). Different letters (a–e) indicate significant difference ($P<0.05$) in MPO release with various stimuli. Asterisk (*) indicates significant difference ($P<0.05$) in MPO release for the same stimulus with or without cyto B.

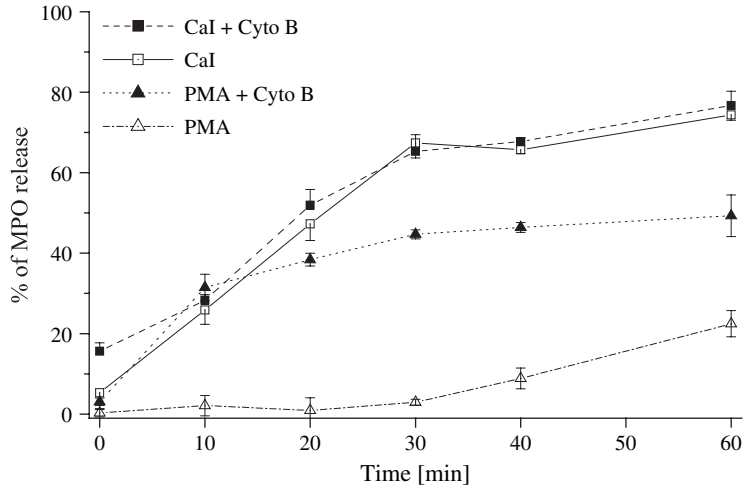

Fig. 4. The kinetics of MPO release from adult fathead minnow neutrophils in response to 5 μg mL^{-1} CaI (rectangle) and 1 μg mL^{-1} PMA (triangle) concurrently exposed with (black) and without (open) 5 μg mL^{-1} cyto B. Release of MPO due to stimulants is significantly greater than in unstimulated neutrophils (not shown) and PMA without cyto B stimulated neutrophils at all time points except 0 ($P<0.05$). Release of MPO due to PMA alone is significantly greater than in unstimulated neutrophils after 30 min. Data are presented as the mean ± SEM ($n=6$).

10 min, except for PMA without cyto B where exocytosis is detectable after 30 min. For both stimulants, the maximum MPO release was recorded at 40–60 min post stimulation, with CaI causing a maximum of 73.1 to 80.3% of total enzyme to be released, regardless of the presence or absence of cyto B. PMA caused significantly less maximal levels of exocytosis both with (49.3%) and without cyto B (22.5%) at 60 min post stimulation (Fig. 4).

3.3. Decrease of exocytosis in stressed fish

The effect of handling and crowding stress, with and without anaesthesia, on neutrophil activity measured as percent of MPO release is shown in Fig. 5. Fathead minnows were exposed to handling and crowding with and without anaesthesia for 20 min and the MPO assay was performed on 0 h, 24 , 72 h and 7 days post exposure using CaI with cyto B as stimulants. There was no significant difference in percentage of neutrophils isolated from the control, S, and SA group fish throughout the experiment. Degranulation did not vary significantly in the control group fish. The overall decrease in exocytosis to 60–75% of control activity in the S and SA groups was observed during the period of 7 days after exposure. A significant decrease in the SA group (75.5% of control group MPO activity, $P<0.05$) was first detected 24 h after exposure while the maximum decrease was observed at 7 days after exposure (60.2% of baseline). No significant difference in MPO activity was seen between the S and SA groups (Fig. 5).

4. Discussion

The MPO assay was originally described to quantitate MPO in human neutrophils for the specific measurement of primary granule exocytosis [15,16]. Also, modifications of the assay have been reported for gilthead seabream (*Sparus aurata* L.) [30]. Granulocyte characterisation in cyprinid species (common carp, *Cyprinis carpio* L. and goldfish, *Carassius auratus* L.) describes the presence of myeloperoxidase in fish

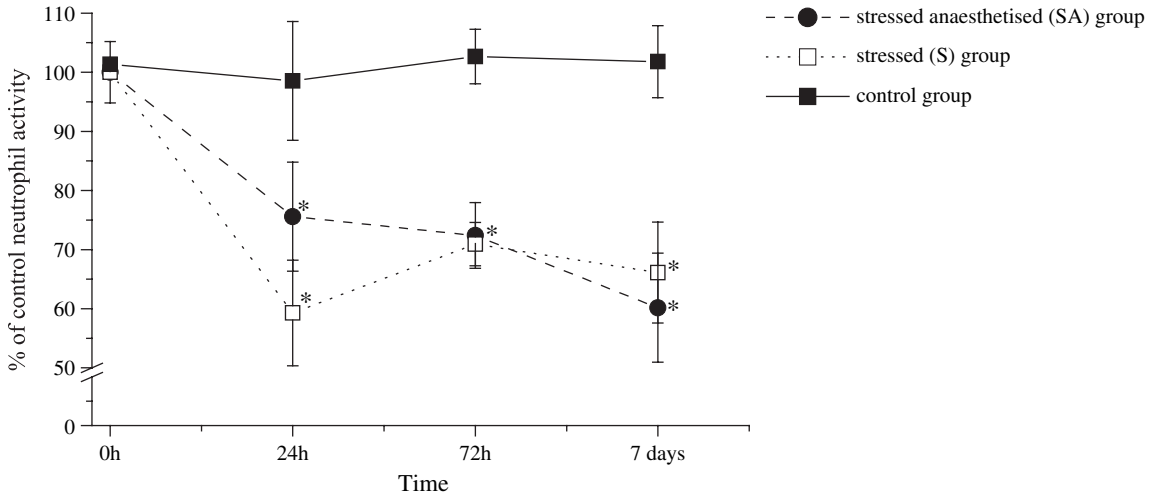

Fig. 5. The reduction of neutrophil activity in adult fathead minnows exposed to handling and crowding stress with (SA) or without (S) 75 mg mL^{-1} of MS-222 for 20 min. At 24 h, 72 h and 7 days post exposure significant decrease in activity was observed in neutrophils from the SA and S groups (*$P<0.05$). There was no significant difference between the SA and S groups. Data are presented as the mean ± SEM ($n=6$).

neutrophils [6,7]. Fathead minnow neutrophils are the most abundant granulocyte cell type in kidney isolates and they contain similar amounts of MPO as bovine neutrophils [31] (2.57 and 2.68 MPO units per 10^6 cells, respectively; Fig. 2). Fathead minnow neutrophils were not disrupted with concentrations of TMB and H_2O_2 used in the assay, and unstimulated neutrophils showed consistently low MPO release (data not shown). Physiologic concentrations of Ca^{2+} appear to be necessary for primary granule exocytosis in fathead minnow kidney neutrophils, since none of the tested stimulants caused a significant release of MPO when resuspended in medium lacking Ca^{2+} with or without cyto B (data not shown). When media with Ca^{2+} was used, neutrophils readily responded to stimulants and the MPO exocytosis was detectable within 1–5 min. This finding is in agreement with studies conducted on human, bovine and seabream neutrophils [12,13,15,16,30].

Cytochalasin B has been reported to be necessary for primary granule exocytosis in bovine, human, and gilthead seabream neutrophils in response to stimulants [16,30,31]. Neutrophils often have been pre-treated with cyto B before being exposed to stimulants, but no significant difference was reported when bovine and seabream neutrophils have been exposed to cyto B and stimulants at the same time [16,30]. The addition of cyto B alone stimulated 11% MPO release in bovine neutrophils [16], but in fathead minnow kidney neutrophils exposed only to cyto B alone, the mean percentage of MPO release was 1.1% (range 0–5%). In this study, it was found that cyto B was necessary for induction of primary granule exocytosis in fathead minnow neutrophils only when PMA was used as the stimulant (Fig. 3). The mechanism of cytochalasin B action on fish neutrophils has not been described, but recent studies of mammalian granulocytes suggest that several exocytosis and degranulation signalling pathways are not all dependent on actin cytoskeletal changes targeted by cyto B [32–34]. This study showed that differences in MPO release were detected in fathead minnow neutrophils exposed to different stimulants. Out of four different stimulants used in this study, CaI and PMA were most effective in inducing neutrophil exocytosis (Fig. 3).

In cattle, human, and seabream neutrophils, degranulation activity in vitro has been shown to be dependent on duration of incubation time [15,16,30]. The kinetic study of MPO release in fathead minnow kidney neutrophils demonstrated that the maximum activity of up to 80% MPO release is reached after 40–60 min when CaI is used as a stimulant with or without cyto B, and stimulation with PMA and cyto B showed significantly less maximum release after 60 min (Fig. 4). In human beings, cattle, and seabream, maximum MPO release by neutrophils (65%, 75%, and 40%, respectively) was detected after incubation of 10–30 min when cells were stimulated with CaI with cyto B [15,16,30]. After 20 min incubation time, mean CaI induced degranulation in fathead minnows was approximately 50% of total MPO release (Fig. 4). Therefore, the MPO assay was responsive to detect changes in fathead minnow neutrophils over time and an incubation of 20 min should be used for CaI as a midpoint time period in MPO release that provides the ability to measure a decrease as well as an increase in exocytosis of primary granules.

It has been recognised that handling and crowding can induce an increased stress response in fish, with an increase in blood cortisol and glucose concentrations [24,25,35,36]. It is known that stress can be a factor leading to immunosupression in mammals and fish [37,38], since corticosteroids act as inhibitors of neutrophil functions, such as phagocytosis, migration and oxidative burst [2,4]. It was demonstrated earlier that MS-222 did not prevent increase in cortisol levels in catfish exposed to handling stress [24]. Also, higher concentrations of anaesthesia induce a decrease in complement activity and phagocytosis of head kidney leucocytes of gilthead seabream [39]. The kinetic study of MPO release in fish exposed to handling and crowding stress with and without MS-222 demonstrated that a decrease in neutrophil primary granule exocytosis in response to stimulation by CaI in the presence of cyto B can be detected at 24 h after exposure, the earliest measured time. The decrease was most significant 7 days after exposure (60% of control) (Fig. 5). Anaesthesia with MS-222 did not prevent decrease in degranulation. No significant changes in percent of kidney neutrophils in the control, S, and SA groups were observed. Therefore, the observed decrease in MPO release from treated fish neutrophils could be related to a possible increase in blood cortisol levels caused by handling and crowding stress.

The MPO assay is direct, quantitative and can rapidly measure a large number of samples, and was shown to be capable of detecting the inhibition of primary granule degranulation and differences in total MPO content between populations of fathead minnow neutrophils. The described assay is a useful method of measuring neutrophil primary granule degranulation and total MPO content in fathead minnow kidney neutrophils and it shows the potential for assessing neutrophil activity during disease states and toxicant exposure.

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