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Short sequence report

Gradient separation and cytochemical characterisation
of neutrophils from kidney of fathead minnow
(*Pimephales promelas* Rafinesque, 1820)

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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 health status [1]. Some literature is non-specific or contradictory about the classification of leucocyte morphology in fish, since morphology alone can vary by species [2–4]. Most studies document that fish neutrophils have very similar morphological and histochemical staining properties to mammalian neutrophils, and can be distinguished by the presence of myeloperoxidase in cytoplasmic granules [5]. Neutrophils are found in fish kidney, spleen and blood, and are commonly increased in inflammatory lesions [6]. There is evidence for phagocytic, chemotactic and bactericidal functions in fish neutrophils and an intense respiratory burst [7–9]. Techniques for obtaining relatively pure neutrophil populations for functional studies have not been previously reported for the fathead minnow (*Pimephales promelas* Rafinesque, 1820), and the characterisation of leucocyte morphology and cytochemical staining has not been adequately defined.

The fathead minnow is a scientifically and ecologically important fish species [10–12], extensively used in toxicology research [13,14], and a significant forage species for larger predator and game fish [15]. In this study, rapid separation of fish neutrophils from the kidney was achieved using a Ficoll gradient yielding an average of 72% neutrophils with a mean viability of 94.4%, as determined by trypan blue exclusion. These

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isolated cells from kidney and similar cells on blood smears were morphologically and cytochemically characterised as neutrophils. Characteristic positive staining reactions for myeloperoxidase and Sudan Black B readily distinguished neutrophils from monocytes and lymphocytes. Positive reactions did not occur in neutrophils when periodic acid-Schiff and alpha-naphthyl acetate esterase staining were performed. Viable intact neutrophils were rapidly isolated from fathead minnow kidney with an acceptable yield and purity for cell function studies. Evaluation of neutrophil function in this animal model will provide valuable information and become a useful tool in ecotoxicology research [16].

Adult fathead minnows with an average weight of 3 g were maintained in accordance with approved Iowa State University animal care guidelines. Fish were sampled in 50 separate trials for isolation procedures and in five separate trials for cytochemical staining procedures. Fathead minnows were caught in a net and immediately killed by immersion in 1 g L⁻¹ MS 222 solution. The caudal peduncle of five fish was severed with a scalpel and whole blood was collected in 50 µL heparinised micropipettes [17]. Kidney and spleen tissue were aseptically collected from 5–20 individual fish, pooled into separate 50 mL test tubes containing 30 mL of Hank's balanced salt solution without Ca, Mg and phenol red (HBSS, Mediatech – CellGro, AK, USA), and homogenised in a 15 mL tissue grinder with 7.5 mL HBSS using 10–15 pestle strokes. Debris from the pestle and the bottom of the tissue grinder were discarded after 2 min, and a portion was used for total and differential leucocyte counts. Then, 7 mL of cell suspension was placed into 15 mL centrifuge tubes containing 5 mL of HBSS, centrifuged (15 min, 250 g), the supernatant discarded, and the viscous mass (resulting from cell debris and release of nuclear material) above the cell pellet was gently removed with Dacron sterile swabs. The cell pellet was resuspended in 6 mL of HBSS with gentle agitation, a portion used for total and differential leucocyte counts, and the suspension gently placed over 5 mL of separation medium with a specific gravity of 1.078 g mL⁻¹ (lymphocyte separation medium 1078, Mediatech – CellGro, AK, USA) in 17 × 120 mm test tubes. Gradients were centrifuged for 30 min, 400 g, and cells that formed a band at the cell suspension, just above the gradient interface, were removed with a 2 mL sterile pipette. Thrombocyte aggregates on the test tube surface were avoided to improve the purity of the neutrophil population. Cells were transferred to a 15 mL test tube, HBSS added for a total volume of 10 mL, and the suspension centrifuged for 15 min, 400 g. Final cell pellet isolates were resuspended in 1 mL of HBSS, and a portion was used for total and differential leucocyte counts, viability determination, and cytochemical staining.

Cell viability was determined by 0.1% trypan blue dye exclusion test. Total leucocyte counts were done using a Neubauer-ruled hemacytometer [18,19], and differential leucocyte counts (>100 cells) were performed using suggested criteria [2,20]. Briefly, neutrophils were identified when the leucocytes contained clear non-staining cytoplasm, moderately coarse nuclear chromatin, and reniform indented or lobulated nuclei with a moderate nuclear to cytoplasmic ratio (Fig. 1C).

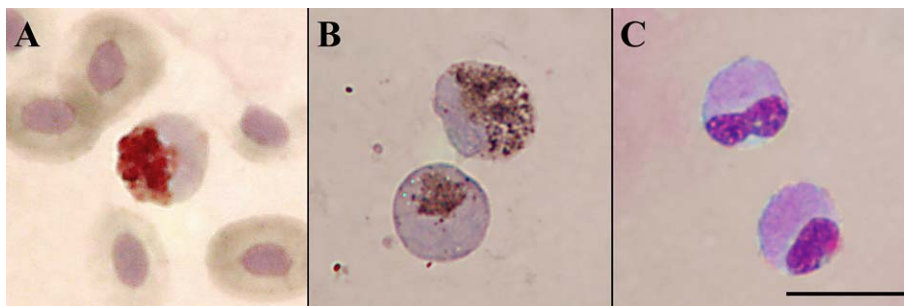


Fig 1. Cytochemical properties and morphology of isolated neutrophils. (A) Characteristic myeloperoxidase positive reaction observed in neutrophils stained with peroxidase N° 391. (B) Characteristic Sudan Black B positive reaction observed in neutrophils. (C) Characteristic morphology of the isolated neutrophils stained with Hemacolor stain. Bar = 10 µM.

Cytochemical staining was performed on whole blood smears, and kidney and spleen cell isolate samples pooled from a minimum of five fish. Canine and bovine blood smears were used as positive controls, and negative controls consisted of slides with the primary reactive reagent eliminated. Commercial kits were used for myeloperoxidase (Sigma, kit N° 391), alpha-naphthyl acetate esterase without fluoride inhibition (Sigma, N° 91A), alkaline phosphatase (Sigma, N° 85L-2), and Sudan Black B (Sigma, N° 380-B) with modified staining times for Sudan Black reagent and haematoxylin stain of 10 and 3 min, respectively. Periodic acid-Schiff staining was performed using a slight modification of a standardised technique [21]. Positive or negative reactions were based on the presence or absence of a cellular chromogen.

The cell band from the kidney contained primarily neutrophils. The first mean total cell yield directly from the kidney homogenates (before purification) was 9.06×10^6 cells/fish (mean neutrophil purity = 25.9%), and following the removal of cell debris prior to gradient purification, the mean cell yield was 5.01×10^6 cells/fish (mean neutrophil purity = 30.26%). The final cell isolation after gradient purification contained $3.29 \pm 2.23 \times 10^6$ cells/fish (range 0.63 to 6.54×10^6 cells/fish), a mean cell viability of $94.39 \pm 2.92\%$ (the majority of nonviable cells were lymphocytes and thrombocytes), and a mean neutrophil purity of $72.0 \pm 7.3\%$. After the separation procedure, the mean total recovery of the neutrophils was 38.97% (neutrophils before separation = 100%). The remaining cells were lymphocytes and thrombocytes, and less than 5% of isolated cells were monocytes, erythrocytes, and a few kidney epithelial cells. Splenic cell isolates contained mostly lymphocytes and thrombocytes with mean total cell yield ranging from 0.73 to 2.22×10^6 cells/fish and a viability of $95.36 \pm 1.1\%$. Low numbers of neutrophils were detected in spleen homogenates before separation (mean neutrophil purity = 5.45%) and after separation (mean neutrophil purity = 9.07%). Eosinophils were rarely identified and no basophils were seen.

Neutrophil granules stained dark red-brown with the myeloperoxidase (peroxidase N° 391) and brown-black with Sudan Black B technique, indicating a positive reaction (Fig. 1A, B). Neutrophil granules did not have a positive staining reaction when alkaline phosphatase or alpha-naphthyl acetate esterase were used, indicating a negative result. Of the various staining methods used, the peroxidase N° 391 staining method and Sudan Black B allowed the rapid differentiation of neutrophils (positive) and monocytes/macrophages (negative) in blood smears and tissue-derived cytospin preparations.

Leucocytes have most often been separated from fish blood and parenchymal organs using continuous and discontinuous Percoll density gradients that require preparation of reagents that prolong cell separation [22]. These methods were not designed to separate neutrophils specifically and yield mixed leucocyte and granulocyte populations [20,22,23]. In the described separation procedure, leucocytes were concentrated at the cell suspension/gradient media interface, and the neutrophil yield was comparable to other species with all nucleated hematopoietic cells [18]. The 70% purity of neutrophil isolates, viability of 95%, and cellular integrity by microscopic examination support the minimal deleterious effects of gradient cell separation on the neutrophils. In addition, the cytochemical staining reactions were similar for blood and neutrophils isolated from tissues, indicating no change in granule or cytoplasmic contents and enzyme activity.

Positive staining of fish neutrophils with periodic acid-Schiff and Sudan Black B procedures has been previously reported [24]. Granulocyte characterisation in cyprinid species (common carp, *Cyprinus carpio* L. and goldfish, *Carassius auratus* L.) describes the presence of myeloperoxidase in neutrophils [3,4]. Minimal data about leucocytes in fathead minnows suggest that neutrophils are the most abundant granulocytes in this species, since no basophils and very few eosinophils have been found in blood smears [25].

Results of this study are in general accordance with published data regarding other fish species. Fathead minnow neutrophils are found to be the most abundant granulocyte cell type in kidney isolates and blood smears with morphological and cytochemical characteristics similar to mammalian neutrophils [1,22,24]. It was demonstrated that fathead minnow neutrophil granules have myeloperoxidase activity, stain positively with Sudan Black B reagent, and are negative for periodic acid-Schiff and alpha-naphthyl acetate esterase.

The described techniques separated semi-pure and viable neutrophil populations from the kidneys of fathead minnows in approximately 3 h for functional studies to provide additional information about fish health status.

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