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Bonnie Bassler and Martin Blaser

to Present Keynote Addresses at the 44th Annual MACUB Conference at Seton Hall University, October 29, 2011

Bonnie Bassler is a member of the National Academy of Sciences and the American Academy of Arts and Sciences. She is a Howard Hughes Medical Institute Investigator and the Squibb Professor of Molecular Biology at Princeton University. Dr. Bassler received a B.S. in Biochemistry from the University of California at Davis and a Ph.D. in Biochemistry from the Johns Hopkins University. She performed postdoctoral work in Genetics at the Agouron Institute, and joined the Princeton faculty in 1994. Her research focuses on the molecular mechanisms that bacteria use for intercellular communication. This process is called quorum sensing. Bassler's research is paving the way to the development of novel therapies for combating bacteria by disrupting quorum-sensing-mediated communication. At Princeton, she teaches both undergraduate and graduate courses.

She directed the Molecular Biology Graduate Program from 2002-2008 and currently chairs Princeton University's Council on Science and Technology which has revamped the science curriculum for humanists. Dr. Bassler is a passionate advocate for diversity in the sciences and she is actively involved in and committed to educating lay people in science.

Dr. Bassler was awarded a MacArthur Foundation Fellowship in 2002. She was elected to the American Academy of Microbiology in 2002 and made a Fellow of the American Association for the Advancement of Science in 2004. She was given the 2003 Theobald Smith Society Waksman Award and is the 2006 recipient of the American Society for Microbiology's Eli Lilly Investigator Award for fundamental contributions to microbiological research. In 2008, she was given Princeton University's President's Award for Distinguished Teaching. She is the 2009 recipient of the Wiley Prize in Biomedical Science for her paradigm-changing scientific research. She is the 2011 recipient of the National Academies' Richard Lounsbery Award.

Dr. Bassler was the President of the American Society for Microbiology in 2010-2011. She is an editor for *Molecular Microbiology*, *mBio*, and Chief Editor of *Annual Reviews of Genetics*. She is an associate editor for *Cell, Proceedings of the National Academy of Sciences, Journal of Bacteriology*, and other journals. Among other duties, she serves on the National Academies Board on Life Sciences, the Howard Hughes Medical Institute Science Education Committee, and Discovery Communications' Science Channel Scientific Advisory Board. She serves on oversight, grant, fellowship, and award panels for the National Academies of Sciences, National Institutes of Health, National Science Foundation, American Society for Microbiology, American Academy of Microbiology, Keck Foundation, Burroughs Wellcome Trust, Jane Coffin Childs Fund, PEW Charitable Trust, the MIT Whitehead Institute, and the Max Planck Society.

The topic of Dr. Bassler's address will be, "*Bacterial Esperanto: The Mechanism Behind Quorum Sensing - or Bacterial Communication.*"

Martin J. Blaser is the Frederick H. King Professor of Internal Medicine, Chairman, Department of Medicine, and Professor of Microbiology at New York University School of Medicine. He is an established researcher in microbiology and infectious diseases. Dr. Blaser's work has focused on *Helicobacter pylori*, *Campylobacter*, *Bacillus anthracis*, and more recently on the human microbiome. He obtained his undergraduate degree from the Univ. of Pennsylvania in 1969, graduated from the New York University School of Medicine in 1973, and did post-graduate training at the Univ. of Colorado School of Medicine from 1973 to 1979. He was an Epidemic Intelligence Service Officer at the Centers for Disease Control and Prevention from 1979 to 1981. In 1998, Blaser established the Foundation for Bacteria. In 2005, he was elected President of the Infectious Diseases Society of America and served the National Institutes of Health on the Board of Scientific Counselors of the National Cancer Institute (2005-2010; Chair 2009-2010), and on the Advisory Board for Clinical Research.

Microbes that are able to persist in their hosts are subject to different selective pressures than those that transiently infect, and either kill their host or are themselves eliminated. In mammals, the gastrointestinal, genital tracts and skin are biological niches that usually are populated by colonizing bacteria. He has been interested in the *Campylobacter* and *Helicobacter* species, highly diverse organisms that live in the mucus layer overlying the mucosal epithelium of mammals, including humans. *H. pylori* colonization increases risk for development of peptic ulcer disease and gastric adenocarcinoma. Conversely, its presence appears to protect against certain diseases of the esophagus. The focus in his laboratory is to explore the biology of *H. pylori* colonization and the nature of the interactions that lead to, or protect from disease. He is examining the variation in particular oligosaccharide (Lewis) antigens on the *H. pylori* cell surface and the nature of the host forces that select for cells of particular phenotypes. Disciplines involved include molecular biology, genetics, and mathematics. He uses transgenic and knockout mice to test hypotheses related to both host factors and bacterial evolution. Other projects relate to restriction-modification systems that act as barriers to horizontal gene transfer, and to a metastable pathogenicity island in the *H. pylori* genome (*cag* island). A third area of work relates to recombination, endogenous mutation, and DNA repair to understand their roles and regulation in the generation of diversity. Another focus of his work is *Campylobacter fetus*, a pathogen of animals and humans. *C. fetus* cells are covered with S-layer proteins that allow the organisms to escape complement-mediated lysis, and undergo antigenic variation. Exploring the molecular basis of variation, he found the S-layer proteins are encoded by a family of *sapA* homologs tightly clustered on the chromosome, and that a high frequency DNA inversion plays a critical role in variation. The inversion shows elements of both site-specific and homologous recombination. This is a highly tractable system to examine DNA recombination mechanisms, as well as for structure-function analysis of protein-carbohydrate (LPS) interactions, and the structural basis of antigenicity. Study of these persistent colonizers has led to explorations of the bacteria (and fungi) normally inhabiting stomach, esophagus and skin using 16SrRNA approaches.

The topic of Dr. Blaser's keynote address will be: *"A mixed Bag: Bacteria That Colonize Humans."*

Instructions for Authors

IN VIVO is published three times yearly during the Fall, Winter, and Spring. Original research articles in the field of biology in addition to original articles of general interest to faculty and students may be submitted to the editor to be considered for publication. Manuscripts can be in the form of a) full length manuscripts, b) mini-reviews or c) short communications of particularly significant and timely information. Manuscripts will be evaluated by two reviewers.

Articles can be submitted electronically to *invivo@mec.cuny.edu* or mailed as a printed copy (preferably with a diskette that contains the file) to the Editorial Board at Medgar Evers College. All submissions should be formatted double spaced with 1 inch margins. The title of the article, the full names of each author, their academic affiliations and addresses, and the name of the person to whom correspondence should be sent must be given. As a rule, full length articles should include a brief abstract and be divided into the following sections: introduction, materials and methods, results, discussion, acknowledgments and references. Reviews and short communications can be arranged differently. References should be identified in the text by using numerical superscripts in consecutive order. In the reference section, references should be arranged in the order that they appeared in the text using the following format: last name, initials., year of publication. title of article, journal volume number: page numbers. (eg. - [1]Hassan, M. and V. Herbert, 2000. Colon Cancer. *In Vivo* **32:** 3 - 8). For books the order should be last name, initial, year of publication, title of book in italics, publisher and city, and page number referred to. (eg. - Prosser, C.L., 1973. *Comparative Animal Physiology*, Saunders Co., Philadelphia, p 59.). Abbreviations and technical jargon should be avoided. Tables and figures should be submitted on separate pages with the desired locations in the text indicated in the margins.

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44th Annual MACUB Conference at
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Morphophysiological Characterization of the Peripheral Blood of Adult Zebrafish (*Danio rerio*)

by

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Abstract

Zebrafish (*Danio rerio*) a small and hardy tropical freshwater fish, which is easily available in all pet stores, has become a popular laboratory animal in genetics and developmental biology. As such it is also becoming widely used for other types of research, for example in studying neuronal degenerative and regenerative processes, a primary focus in our laboratory. Neuroregeneration is aided by different, specific non-neuronal cells like glial cells. However, other non-neuronal cells, such as different white blood cells and their derivatives also appear concentrated in the field of degeneration/regeneration. In order to be able to recognize the activated *in situ* blood-derived-cells under transmission electron microscopy, ultra structural characterization of such cells became a necessity. Accordingly, in this work we describe all classes of the formed elements of the peripheral blood in adult zebrafish using light and transmission electron microscopy, compared to mammalian (human) formed elements.

Introduction

During the last two decades, zebrafish (*Danio rerio*) became a widely used laboratory animal in genetics and developmental biology[1]. The reason for the rapid success of zebrafish in these types of research is the animals' low price and easy maintenance; small size, high fecundity, non-placental embryogenesis external to mother within a transparent chorion during which development is independent of maternal care, and its short developmental cycle. The ever-growing knowledge about gene expression and development of zebrafish is turning these animals into nearly equivalent-ones to mice and rats in other types of experimental research as well[2]. Our laboratory also uses adult zebrafish as subjects for studying nervous tissue degeneration and regeneration after traumatic brain injury (TBI) from a neuroimmunological perspective. Immunity not only relates to the body's struggle against infections but also plays an important role in the maintenance of homeostasis and in regeneration after injury. It is known that white blood cells and globulin type plasma proteins are the main constituents of the immunity of an organism. In order to better understand the degenerative/regenerative processes that take place in injured zebrafish brain, understanding the morphological and physiological characteristics of the zebrafish peripheral blood became imperative.

According to literature, the zebrafish turned out to be a suitable model organism for hematopoesis and it has been found to be similar to that in mammals and other higher vertebrates whose representative blood cell types include the erythroid, thrombocytic, myeloid and lymphoid cell lineages[3]. Although hematopoiesis in zebrafish is considered well studied, adult zebrafish blood has been mostly neglected and studied so far by only few investigators such as Jagadeeswaran *et al.*[4], Hsu *et al.*[3] and Crowhurst *et al*[5]. Apart from some basic information available regarding adult zebrafish blood, still little is known about its basic morpho-physiological characteristics, such as the ultrastructural features and physiological specificity of blood cells, among others[6]. For this reason, and because it is important for us to record white blood cell activities during TBI, we decided to study and characterize adult zebrafish blood with special focus on the white blood cells.

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Materials and Methods

Animals used and Collection of Peripheral Blood

Adult zebrafish (*D. rerio*) were obtained from a local pet store and housed in a 40 gallon tank with ambient lighting conditions at room temperature and fed with regular fish flake food. Prior to blood collection, fish were anesthetized with a 0.04% tricaine solution for one minute. Gills were removed to gain access to the heart, a small incision was made into the ventricle and the blood was harvested using a 0.1% heparinized capillary tube. After collecting the blood, the injured fish were frozen at -20°C before discarding. The harvested blood was used to prepare smears or collected in a micro-centrifuge tube with fixative to be processed for light and electron microscopy.

Blood Smears Staining

Blood smears were prepared on glass slides, air-dried and fixed in absolute methanol for 10 minutes. For staining a 1:1 diluted May-Grünwald solution (Electron Microscopy Sciences Co.) was used for 5 minutes followed by a 1:9 diluted Giemsa solution (Ricca Chemical Co.) for 30 minutes at room temperature. Dilutions were prepared in distilled water. Fixed blood smears were also stained using Wright-Giemsa staining solution for 30 seconds. Following the staining, the slides were washed with distilled water, air-dried and coverslipped. The slides were then analyzed and cells were photographed using an Olympus BX40 light microscope equipped with a Motic 2500 moticam digital camera. Microphotographs were captured using Motic Images Plus software and plates were assembled using Adobe Photoshop CS3 software.

Transmission Electron Microscopy (TEM)

For transmission electron microscopy blood from 4-7 fish was collected in a micro-centrifuge tube with Karnovsky's fixative solution (4% paraformaldehyde and 2.5% glutaraldehyde) for 10 minutes and centrifuged for another 10 minutes at 13K rpm. The pellet was washed and centrifuged twice with 0.1M phosphate buffer (pH= 7.2) for 5 minutes then post-fixed with 1% osmium tetroxide for 10 minutes and centrifuged for another 10 minutes. The pellet was washed and dehydrated through growing concentration of ethanol (50%, 70%, 95% and 100%). The dehydration with 100% ethanol, as well as with propylene oxide, was changed twice for 5 minutes each. Spurr was used as the embedding medium (Electron Microscopy Sciences, Co). Prior to embedding, solutions of Spurr in propylene oxide were prepared in different concentrations (1:2, 1:1 and 2:1), added to the pellet and centrifuged for 10 minutes. Spurr was then added to the pellet, the micro-centrifuge tube was sealed with parafilm and incubated overnight at 4°C. The following day the pellet was embedded and incubated overnight at 70°C for polymerization. Polymerized blocks were trimmed and sectioned on a Sorvall MT6000 ultramicrotome using glass knives. Semithin sections were collected on slides and stained with toluidine blue. Ultrathin sections were collected on 200 count copper mesh grids and stained with lead citrate and saturated uranyl acetate for 8 minutes each. Grids were studied using a Philips CM-100 transmission electron microscope and photographed with a *Motic 2500* digital camera.

Note: Stained and unstained blood smears were also sent out to two different diagnostic centers to obtain professional opinion concerning the accuracy of cell classification: (1) the Animal Health Diagnostic Center at Cornell University (Ithaca, NY) and (2) Hemopet/Hemolife (Garden Grove, CA). Their reports were used as some standard information on determining cell types and their numbers.

Results

The goal of this study was to characterize the cell types in adult zebrafish peripheral blood using transmission electron microscopy. Prior to the electron microscopic analysis, classical staining techniques were employed in order to orient ourselves as to the cell morphology in semithin sections and then in electron microscopy. Two different approaches at light microscopic level were used, namely – blood smears stained with classical blood dyes and semithin sections stained with toluidine blue. The semithin sections utilize embedding procedures classically used for transmission electron microscopy and as such allows for a better understanding of the transitional forms from light to electron microscopy in sections. Moreover, for help, the cell types of zebrafish blood were compared with the well studied cell types, - their mammalian counterparts, - using human blood (blood samples from Z. Franco). Below the findings are described, grouped around the techniques used and by specific cell types.

Light Microscopy 1: Classical stain

The blood smears were stained with several different staining techniques, such as Wright, Wright–Giemsa and May Grünwald–Giemsa.

However, the May Grünwald–Giemsa stain turned out to provide the best coloration. In this dye, the basic dye methylene blue and the acidic dye eosin are combined into a "neutral" solution, which produces a wide color range when staining. The pH of the staining solution is critical and ideally should be adjusted for different fixatives. More acidic pH levels give more selective chromatin staining and less cytoplasmic basophilia; less acidic pH levels give denser nuclei and increased cytoplasmic basophilia. To get a proper staining, the pH range should be between 6.4 and 6.9. With this stain, the nuclei stained with varying shades of purple, while the cytoplasm varied in shades from light pink to reddish, and cellular granules varied from whitish to bluish shades.

As an aid in analyzing the cell type's morphology, many individual cells were photographed with a 100x oil immersion objective, photographs were grouped based on the cells' major morphological characteristics and size, and these groups were matched with literature for teleosts data and with similar groups in human blood. Names used for the different cell types were taken from Campbell and Ellis, 2007[7].

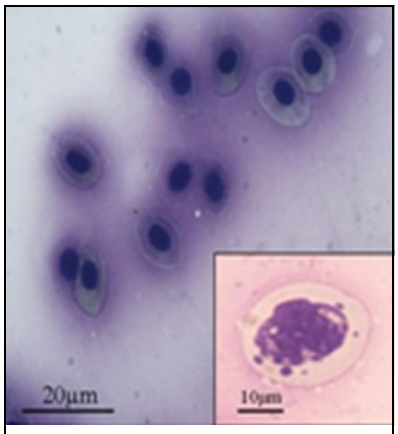

Figure 1. Red blood cells and thrombocytes as they appear with May-Grünwald-Giemsa stain in light microscope under 100x oil immersion objective.
Scale bars = 20µm and 10µm

Erythrocytes and thrombocytes

Erythrocytes in zebrafish (Figure 1) differ from mammalian erythrocytes by the fact that they are nucleated in their mature form. Erythrocytes stained with May Grünwald–Giemsa solution showed oval to round shape with a centrally located nucleus. They actually possess a large nucleus staining deep purple. The cytoplasm of erythrocytes would either stain pale purple or remain unstained. They may appear singly or in clusters.

Zebrafish blood does not contain platelets; instead they have thrombocytes, complete, nucleated cells. The round to oval, elongated or spindle-shaped cells with deep blue nuclei were recognized as thrombocytes (Figure 1, inlet), Thrombocytes may show several cytoplasmic processes giving to this cell type a "dendritic" appearance. Thrombocytes are cells that play a key role in blood clotting in non-mammalian vertebrates and are functionally similar to the anucleated cell fragments called platelets in mammals.

Agranulocytes

Monocytes in mammalian blood have two main known functions: (1) replenish resident macrophages and (2) respond to injury. Monocytes can move quickly (in 8-12 hours) to sites in the tissues and divide/differentiate into macrophages to phagocyte debris and elicit an immune response. Monocytes are usually identified in stained smears by their large, slightly bilobated, bended nucleus. Monocytes in zebrafish blood (Figure 2, first row) were recognized as cells with agranular cytoplasm and their bean-shaped nucleus stained dark blue or purple with a cytoplasm stained with pale shades of the same colors.

Lymphocytes (Figure 2, second row) were seen as round cells with varying sizes having round nuclei and small cytoplasmic rings. The nuclei stained deep violet, while the cytoplasm was either a light blue ring or, in some cases, not visible at all.

Granulocytes

Basophils (Figure 3, first row) were found as round, granulated cells with an irregular outline and a large nucleus. In some cases it was difficult to distinguish the nucleus because of the heavily stained granules that filled the cytoplasm. These cells stained dark blue or purple frequently showing bright spots in their cytoplasm as signs of degranulation.

The eosinophils (Figure 3, second row) observed were round cells with a round, sometimes eccentrically located nucleus stained purple/pink. Its cytoplasm stained pale to dark purple with abundant pink/purple shining granules.

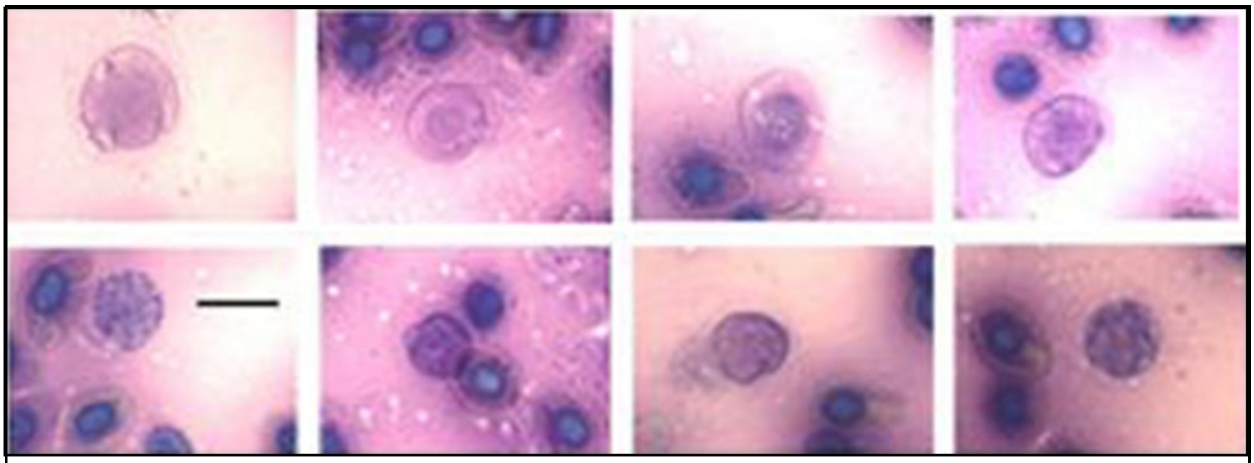

Figure 2. The first row depicts monocytes of different sizes and stages. The second row depicts lymphocytes. The differences between the morphology of these cells may be due to being B or T lymphocyte. Scale bar = 20μm

Neutrophils (Figure 3, third and fourth rows) were present as round/oval cells with granulated cytoplasm and eccentric nuclei, which may appear lobed. Their nucleus stained a red/violet color, while the cytoplasm was either a blue or purple ring in some cases not visible.

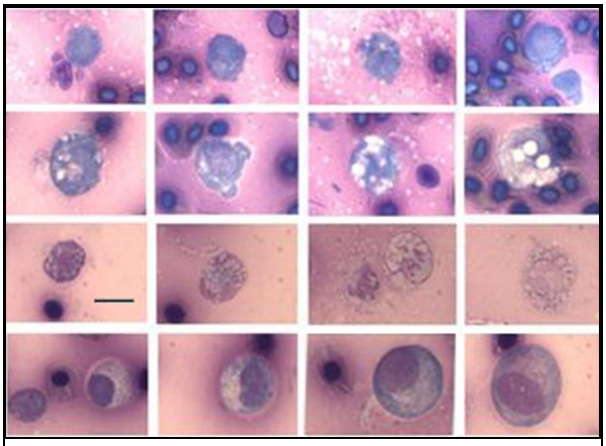

Figure 3. The first two rows depict basophils of different size, shapes and activity. The third row depicts eosinophils of different sizes and stages. The fourth row depicts neutrophils. Scale bar = 20μm

Light Microscopy 2: Semithin sections stained with toluidine blue

Semithin sections are often used in transmission electron microscopy to aid recognition of certain cellular characteristics seen in light microscopy. This is possible because semithin sections are cut from the same embedded tissue sample used for the ultrathin sections. Semi thin sections, stained with toluidine blue, give a good general overview of the sample under high power light microscopy, somewhat similar to that of a low magnification electron microscopic field.

In this case, as it was with the classical stains, many cells were photographed and then grouped based on their major morphological characteristics and size. The morphological characteristics of red and white blood cells of the adult zebrafish blood are presented below by categories.

Erythrocytes and thrombocytes

Erythrocytes (Figure 4) in zebrafish are disk-shape cells with a relatively large nucleus in the center. The shape of the nucleus can be ellipsoid or spherical. Typically both the nucleus and the cytoplasm appear to be homogeneous, but nuclei with differentiated chromatin can also be detected. The unstained cytoplasm appears somewhat reddish but turns into light blue after staining. As the cells fall during centrifugation in many different orientations, in section they show several different profiles, from ovoid to round (Figure 4A) or spindle -like (Figure 4B) and anything in-between.

Thrombocytes show ovoid to round nuclei which are smaller than those of the erythrocytes. The cytoplasm of thrombocytes has few or many cytoplasmic protrusions, giving them a "dendritic" appearance (Figure 4B, all inserts).

White blood cells in blood smears are typically recognized by the presence or absence of granules. Granulocytes are further differentiated based on the chemical nature, as well as size and appearance of granular content. In case of semi-thin sections stained with toluidine blue, these cells are harder to identify. The uniform blue stain does not allow differentiating between the chemical natures of cytoplasmic granules. Furthermore, due to sectional presence of only a

portion of the cell, granules may frequently be missing from the section. However, better resolution of the different nuclear and cytoplasmic content may counterbalance this problem. Also, as it was stated above, it represents a link between

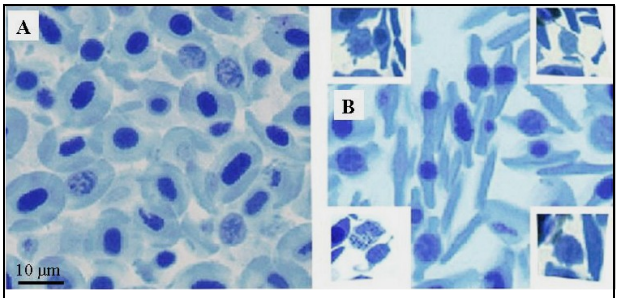

Figure 4. A depicts erythrocytes sectioned parallel to their larger surface and the cytoplasm is either ovoid to round shape; In B, the section shows a profile of the cell and the cytoplasm appears spindle-like. Erythrocyte nuclei appear either as dense, homogeneous substance or showing a lattice-like combination of eu- and hetero-chromatin. The inlets in the right photo show different thrombocytes.

light and electron microscopic analysis of these sectioned cells.

Monocytes and Lymphocytes

In semithin sections the nucleus of lymphocytes appears dense, round or irregular shape and surrounded by a small ring of light-blue cytoplasm (Figure 5). The cytoplasm, however, may contain small, darkly stained granules or somewhat larger, whitish vacuoles. Monocytes in sections look very much like lymphocytes. The size of the sectional profile is irrelevant to the actual size of the cell, so the only morphological difference between the two cell types are the nuclear chromatin and the shape of the nucleus, which is frequently bean or kidney shaped. The chromatin is characteristically divided up for eu- and hetero chromatin.

Basophils, Eosinophils and Neutrophils (Heterophils)

In semi-thin sections the nucleus of basophils are located eccentrically and may appear dense and lobated with an irregular shape, surrounded by a large cytoplasmic area. The cytoplasm contains large, rounded granules which in some cases stain dark-blue and in others they may appear as bright spots (Figure 6, top row). The eosinophils contain cytoplasmic granules of similar appearance to that of basophils, but they are somewhat larger and elongated (Figure 6, middle row). Neutrophils in semi-thin sections show multi-lobated (usually 2 or 3), eccentric nucleus not much darker than the cytoplasm. Their cytoplasm stains light blue and has a dense appearance as a result of the lightly stained, small granules (Figure 6, bottom row).

Transmission Electron Microscopic identification of the different blood cell types aided with colored light microscopic images and compared with mammalian cells

The Red Blood Cells (RBCs) of the human blood (Figure 7A,B) have no nucleus; they have an average diameter of 7 µm and about a 2-3 µm thickness. They have a biconcave, donut shape and are filled with hundreds of millions of hemoglobin molecules that are evenly distributed giving the cells their typical amorph looking appearance. The platelets are small vesicle-like structures filled with different proteins. RBCs in the zebrafish blood (Figure 7C,D) have nuclei. They have a biconvex, ellipsoid shape and are filled with hundreds of millions of hemoglobin molecules that are evenly distributed giving the cells their typical amorph looking appearance. The zebrafish have no platelets, instead they have nucleated thrombocytes, which are smaller than RBCs.

Lymphocytes are the most numerous white blood cells in the human blood (Figure 8A,B). They are small, round cells, with diameters between 4-7 µm. They have a dense nucleus surrounded by a thin brim of azurophilic cytoplasm. They may show some pseudopods and very small eosinophilic cytoplasmic granules. The differences in their appearance may be due to their stage of development or level of activation. Lymphocytes, also the most numerous white blood cells in the zebrafish blood, resemble in their appearance the lymphocytes of other vertebrates, including humans (Figure 8C,D). They are small, round cells, around 5µm in diameter with a dense nucleus surrounded by a thin brim of azurophilic cytoplasm. They may show some pseudopods and very small eosinophilic cytoplasmic granules.

Monocytes in the human blood resembles large lymphocytes with no or very small cytoplasmic granules and possible pseudopods (Figure 9A,B). However, their nucleus assumes a bean shape, sometimes divided even further into three lobes. The average size of the cells is around 15 µm. When migrating into the

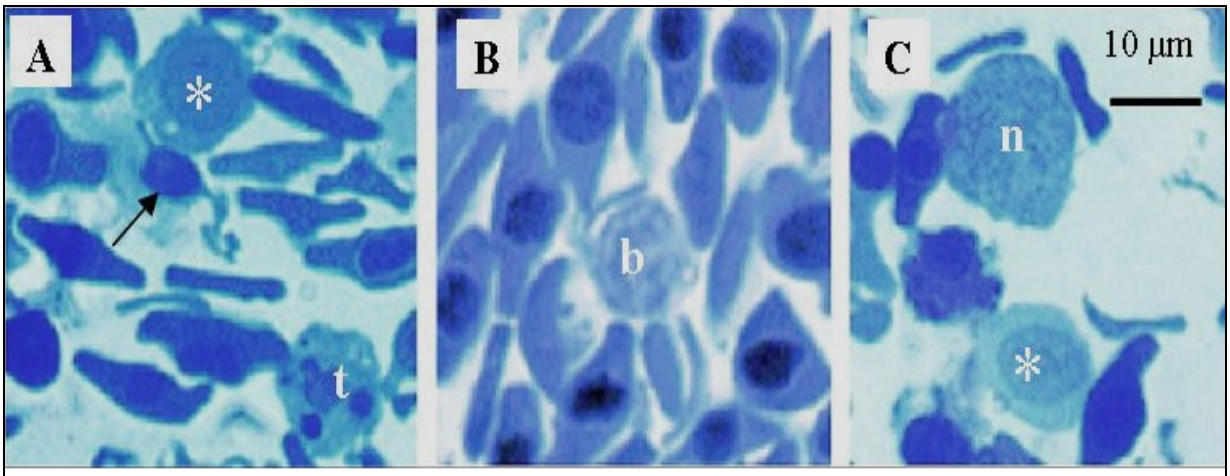

Figure 5. In these three micrographs we show two monocytes (*) and a lymphocytes (arrow). Also seen are: RBCs; a thrombocyte (t), a basophil (b) and a neutrophil (n).

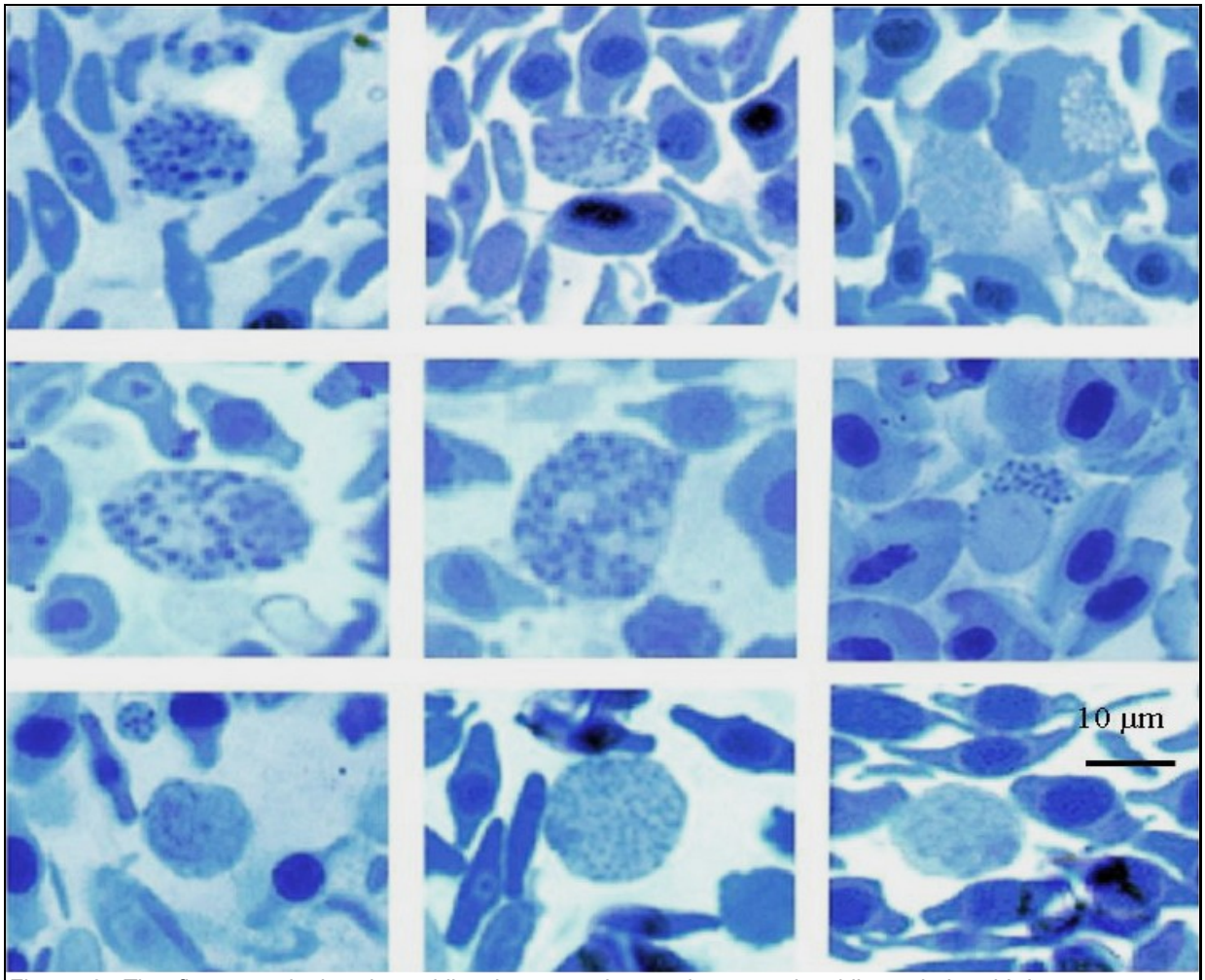

Figure 6. The first row depicts basophils, the second row shows eosinophils and the third row presents neutrophils.

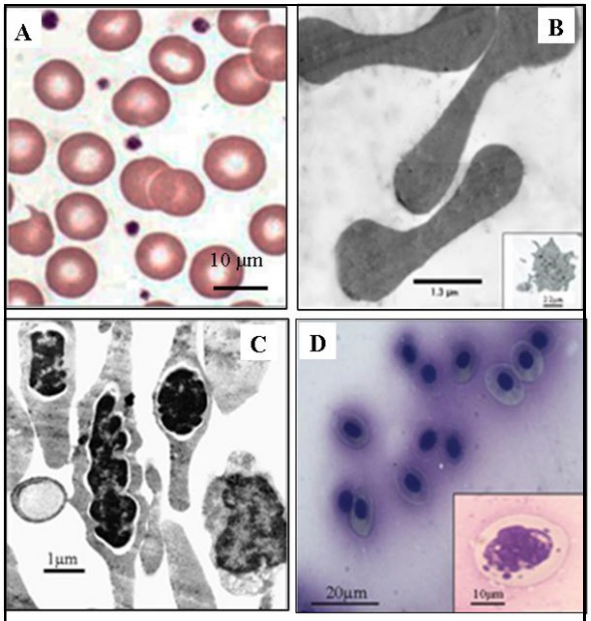

Figure 7. Red blood cells, platelets and thrombocytes. A and B Human blood (The insert in picture B is a platelet). C and D Zebrafish blood (The insert in picture D is a thrombocyte).

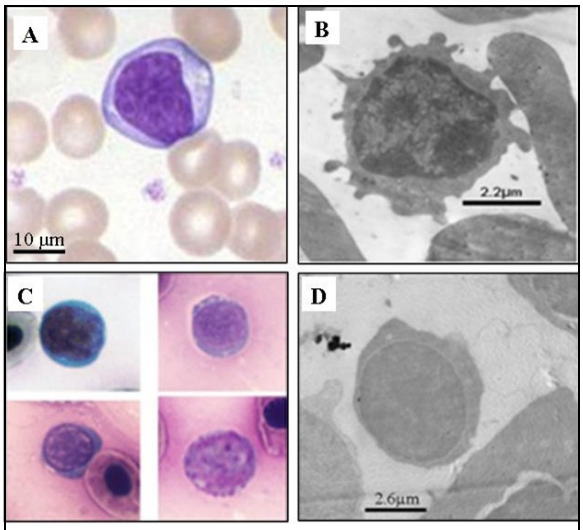

Figure 8. Lymphocytes. A and B -- Human blood; C and D --Zebrafish blood.

surrounding tissues they become macrophages, the largest phagocytic cells. Monocytes in the zebrafish blood are large, mono-nucleated white blood cells with an average size of 15-17 µm (Figure 9C,D). The nuclei of the zebrafish monocytes are scarcely indented and only in few cases show bean shape. Small cytoplasmic processes and some small granules could occasionally be seen.

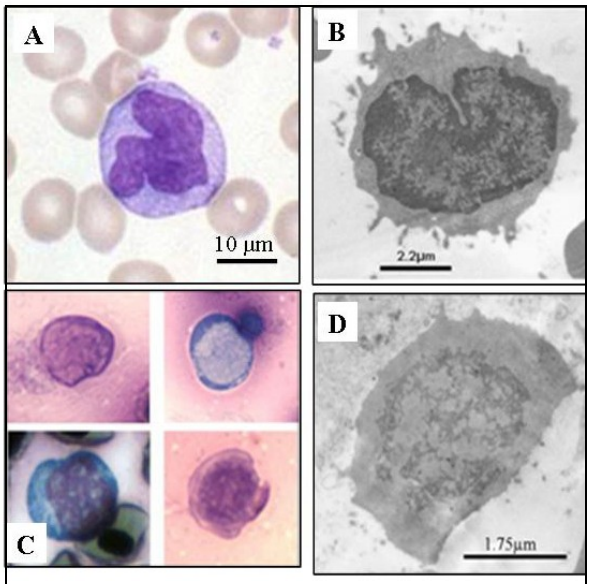

Figure 9 Monocytes. A and B -- Human blood; C and D --Zebrafish blood.

Basophils in human blood are easily recognized by their round to oval, basophilic granules and the bean-shaped nucleus (see TEM picture, Figure 10A,B). The nucleus, however, may be multi-lobated (LM picture). They make up less than 1% of the WBCs in the peripheral blood and frequently migrate through the walls of capillaries and small venules into the surrounding tissues where they are present as mast cells. These types of cells are believed to have different regulatory molecules such as histamine, heparin and serotonin which are released by "degranulation".

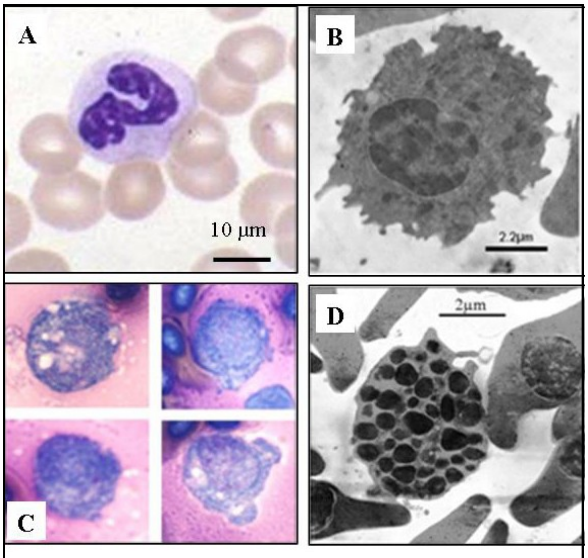

Figure 10. Basophils. A and B -- Human blood; C and D --Zebrafish blood.

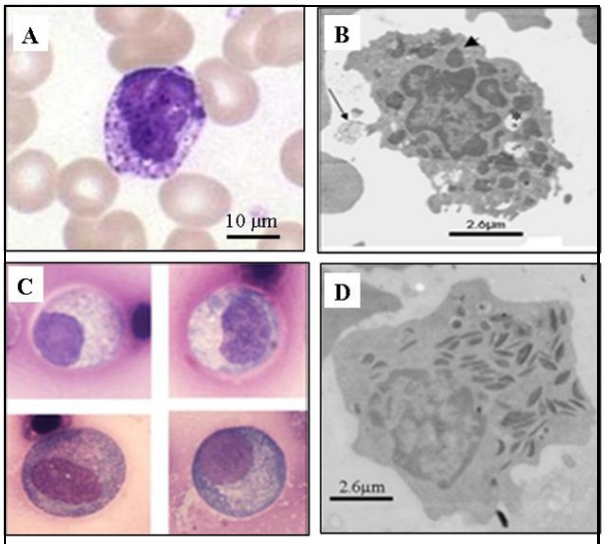

Figure 11. Eosinophils. A and B -- Human blood; C and D --Zebrafish blood.

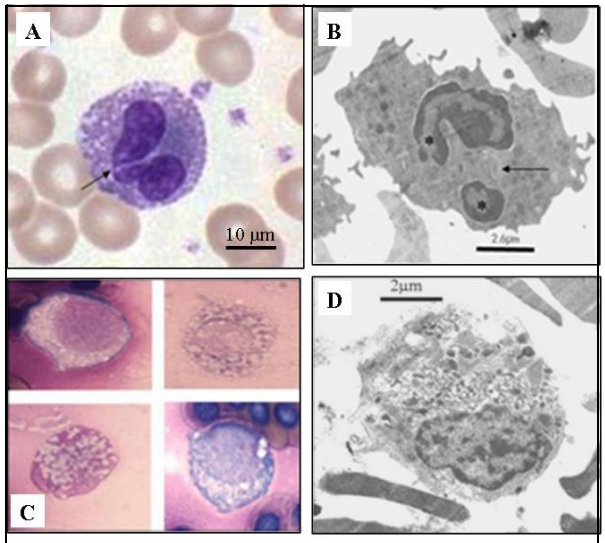

Figure 12. Neutrophils. A and B -- Human blood; C and D --Zebrafish blood

Basophils in zebrafish blood measures between 10-20 µm (Figure 10C,D). They are rounded cells with a rounded, eccentrically located nucleus with homogeneous chromatin and numerous, large basophilic or metachromatic cytoplasmic granules known to contain heparin and serotonin, but lacking histamine. After migrating into tissues they turn into aggressive phagocytes, referred to as macrophages.

Eosinophils in LM are recognized by their large eosinophilic granules, which in TEM appear as large, electro-dense inclusion, known to contain some electron lucent crystalloids (asterisk and arrowhead, Figure 11A,B). Vacuole-like spaces with inclusions indicate phagocytosis. This cell type contains several enzymes, which are believed to be released (arrow) to neutralize the agents secreted by mast cells. The nucleus is bi– or tri-lobed with a mixture of hetero and euchromatin.

Eosinophils in zebrafish blood closely resemble the eosinophils of other vertebrates, including humans Figure 11C,D). They are less numerous than the other granulocytes, but can be easily distinguished by their typical, rod or rounded shaped eosinophilic granules. TEM reveals that the eosinophil's cytoplasm may extend out as short processes toward some extracellular structures, a sign of possible phagocytosis. The nucleus is slightly indented and filled with even mixture of hetero and euchromatin. Typically the nucleus is located eccentrically.

Neutrophils in human blood are recognized by their multi-lobated nuclei with typical nuclear "bridges" (arrow), which are chromatin-free doublings of the nuclear envelope (Figure 12A,B). In TEM the chromatin shows characteristic centrally located homogeneous euchromatin surrounded by homogeneous heterochromatin (asterisks). The cytoplasm contains lysosomes. Protrusions of the cytoplasm indicate phagocytic capacity. Heterophils (or neutrophils**)** are the more numerous granulocytes in the zebrafish blood and they resemble mammalian neutrophils Figure 12C,D). They have small to large granules, some vacuole-like inclusions and pseudopods, typical features of a phagocyte. The granules stain with either acidic or basic dyes (thus the name: heterophils). The eccentric nucleus is non-segmented but shows slight indentions. The euchromatin and few heterochromatin islets are surrounded by dense peri-nuclear heterochromatin.

Discussion

Teleost fish and its hematology

Approximately 450 million years of evolutionary divergence separates mammals from bony fish (teleosts). Today teleosts account for nearly half of all known vertebrate species. Teleosts have mastered life in water, colonizing virtually all aquatic systems. Data suggest that the spleen in adult teleost functions as a reservoir of erythrocytes and a site of their destruction. Erythrocytes serve the familiar function of oxygen transport and presumably also in metabolic regulation as in mammals[6]. Macrophages are

present in the adult fish kidney and spleen, organs that also contain erythrocytes and other cellular debris. Like mammals, teleosts possess a separate macrophage lineage and several types of granulocytes[5]. The adult teleost site of granulopoiesis is the kidney interstitium. In the literature, the most abundant fish granulocyte has variously been called a "heterophile" or "neutrophilic" granulocyte, a cell type that's referred to as neutrophile in mammals. The phagocytic activity of neutrophils in zebrafish remains to be determined. These are characteristics shared by the different species of the teleost group including the zebrafish (*D. rerio*), the model organism for this research[8].

The zebrafish, *D. rerio*, is a tropical freshwater fish belonging to the Cyprinidae family. The zebrafish is native to the streams of the southeastern Himalayan region, including the countries Pakistan, Bangladesh, Nepal, and Myanmar, but it has also been found in Colombia[9]. It commonly inhabits streams, canals, ditches, ponds, and slow-moving to stagnant water bodies, including rice fields. The fish is named for the five uniform, pigmented, horizontal blue stripes on the side of the body, all of which extend to the end of the caudal fin. Its shape can be described as fusiform and laterally compressed. Males are differentiated by the gold stripes between the blue stripes; females have a larger, whitish belly and have silver stripes instead of gold. The zebrafish can grow up to 6.4 cm (2.5 in), although it is uncommon for them to grow past 4 cm in captivity[10].

Zebrafish hematology

Zebrafish is an important vertebrate model organism in scientific research, which has contributed to hematologic research for more than 50 years. Interest in zebrafish embryology dates to the 1930s, and they have long been used for zoology and toxicology research. The first description of zebrafish blood, cell morphology appeared in the 1970s[6].

Peripheral blood smears of zebrafish have demonstrated circulating erythrocytes, granulocytes, monocytes, lymphocytes and thrombocytes. However, because of the small size of zebrafish, quantification of normal adult hematologic indices is not as straightforward as in larger species. Wide discordance in the reported relative proportions of leukocytes types may reflect technical differences in blood collection and smear preparation.

Haemostasis is a vertebrate specific function that is critically important for the response to vascular injury and development of human thrombosis. In mammals haemostasis represents formation of the initial platelet plug, by an integrated process, which includes components of adhesion, activation, aggregation and secretion.

Haemostasis in fish is regulated by thrombocytes, which are nucleated cells that are smaller than erythrocytes, and have variable shapes and cytoplasmic projections. The presence of nucleated thrombocytes differentiates zebrafish from mammals, although they have been found to function analogously to platelets in adherence, secretion and aggregation. It has also been discovered that zebrafish possess all the major proteins of the coagulation system when compared to the same system in humans[11]. Thrombocytes act in response to an injury in the clotting process, which usually occurs within 5 minutes in fish. However, fish appear to rely primarily on extrinsic pathways of coagulation, because of the high calcium solutions, sea water or other extrinsic factors that enhance clotting[12].

Erythropoiesis in the adult zebrafish takes place in the interstitium of the anterior and posterior kidneys[6]. Erythrocytes of zebrafish are similar in appearance and ultra-structure to those of birds and reptiles. Mature erythrocytes are oval to ellipsoidal with abundant pale eosinophilic cytoplasm and centrally positioned oval to ellipsoidal nuclei. Immature erythrocytes (1% of the red cell population) may be noted in normal blood films of zebrafish because erythropoiesis occurs in the peripheral blood[12-15]. Immature erythrocytes appear as round cells containing less cytoplasm than mature erythrocytes. Their nuclei is larger than the nuclei of the mature cells, they appear as rounded and less condensed in comparison to the nuclei of mature erythrocytes. Regardless of their morphological differences zebrafish erythrocytes serve similar functions of oxygen transport as mammalian erythrocytes. Histology suggests that the zebrafish adult spleen functions as a reservoir of erythrocytes and a site of their destruction, rather than as a lymphoid organ[6].

Myelopoiesis in zebrafish generates monocytes and several, if not all types of granulocytes [16]. As in mammalian agranulocytes, white blood cells are also found in the peripheral blood. Monocytes are typically the largest leukocyte found in the peripheral blood of zebrafish and they resemble the monocytes of birds and mammals. They appear as large

mononuclear leukocytes with agranular cytoplasm that may present pseudopod-like structures. And their nuclei may vary from kidney-shape to partially lobed. Monocytes are actively phagocytic cells and participate in acute inflammatory responses[12]. Lymphocytes play a major role in the humoral and cell-mediated immunity of zebrafish and other fish[17].

Adult zebrafish contain 2 distinct granulocytes, a heterophil/neutrophil (presumed to be functionally homologous with the mammalian neutrophile) and a rarer eosinophile, both of which circulate and are generated in the kidney, the adult hematopoietic organ[16]. Basophiles are rare in peripheral blood of zebrafish and can be sometimes confused with mast cells[5].

Neutrophils have been found to be the predominant granulocyte of zebrafish blood. Adult zebrafish neutrophils, like their human counterparts have segmented nucleus and granular cytoplasm. However, the nuclei of zebrafish neutrophils are divided into two or three lobes rather than the four or five lobes typical of mammals[3]. The extent to which adult zebrafish neutrophils are phagocytic is unknown and remains to be determined[6].

Eosinophilic granulocytes have also been observed in zebrafish peripheral blood in low concentrations. Eosinophiles can easily be differentiated from other granulocytes by their round to rod shaped granules in the cytoplasm [7]. Although its function has not been yet demonstrated, some reports indicate that they participate in inflammatory responses, usually parasitic infections or antigenic stimulation, along with heterophils/neutrophils and macrophages and appear to have limited phagocytic capability[12, 13].

The last type of granulocyte is the basophil, which is rarely seen in peripheral blood of zebrafish and when it is seen it occurs in very low numbers. They are identified as round cells similar in appearance to mast cells, with round granules[12]. The presence of the basophil in the fish's blood has not been associated with any recognized disease process, making its function unclear. It has been found that mast cells as well as basophils, in fish blood lack histamine, which is commonly the main vasoactive component of avian and mammalian mast cells and basophils[13].

Several classical blood stains were tried but it was found that the May-Grünwald- Giemsa stain was the most informative in recognizing the different cell types of the zebrafish blood. Accordingly the WBC's reported here were stained with the May-Grünwald/Giemsa technique. It was found that zebrafish blood contains all the WBC's types which are typical in vertebrate animals, namely granulocytes (basophils, eosinophils, neutrophils) and agranulocytes (monocytes and lymphocytes). However some unique characteristics were observed, precisely that the granulocytes are not polymorphic, in other words they are not lobated. Only bean-shaped nuclei were observed occasionally. The RBC's of the zebrafish blood show ellipsoid flatten shape with large nucleus in the center that shows characteristic chromatin arrangement. Interestingly it was observed that, in some individual fish blood samples some of the RBC's are "giant", sometimes 2 to 3 times larger than typical RBC's. Another interesting characteristic is that many RBC-looking cells appear as "ghost", non-nucleated cell such as the red blood corpuscles of higher vertebrates. Since we were not able to detect structures that would remind us of platelets we believe that the larger looking RBC might be thrombocytes, typical for lower vertebrates. As for the non-nucleated RBC's as a possibility, only one published article was found, that mentions such cells. Results of this study show several similarities yet also some differences between the light and electron microscopic morphology of human and zebrafish mature blood cells. Both red blood cells (RBC's) and thrombocytes in zebrafish are nucleated cells carrying some morphological characteristics of white blood cells (WBC's). The impression after observing the zebrafish WBC's has been that all types, both the granulocytes and agranulocytes contained pseudopods or cytoplasmic process as well as lysosomes or vacuole-like structures with inclusions, suggesting that they perform phagocytic activities. Several cell types showed different, electron-dense or lucent granules and signs of degranulation or active release of cellular products indicating that these cells perform regulatory functions as well. Further studies aim to investigate functional differences using adequate physiological techniques.

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Comparing Tests of Anatomical Knowledge Before and After the Course: Evaluation of Critical Thinking Skills

by

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Abstract

Critical thinking skills are essential to succeed in today's complex world. In order to improve and retain these skills in an upper level science course, methods of higher cortical processing are modeled in the classroom, including working with case studies. By using a numerical scale to compare the answers of nine anatomically-related questions, on tests administered the first and last day of lecture, critical thinking data was evaluated. Descriptive and comparative statistics revealed that students significantly improved critical thinking after the semester of instruction.

Introduction

Critical thinking, higher cortical processing, has previously been defined as possessing certain skills that reflect an ability to evaluate situations or problems in an informative manner[1], and using traditional and unorthodox thought strategies that are ultimately purposeful, reasonable and goal-directed[2]. Critical thinking skills, abilities that center on systematic analysis of complex questions while being receptive to various schemes to achieve solutions, are essential to succeed in today's complex world. Demonstrating various models of higher cortical processing in the classroom increases the likelihood that students will practice these paradigms that incorporate open-mindedness and systematic analysis[3]. According to Lederer[4], teachers can strengthen critical thinking skills by: repetitively asking questions during lecture, explaining what is being sought when a question is posed to the class and what the correct answer(s) demonstrate about the depth of knowledge; and actively engaging students to elaborate upon answers to questions, beyond the simplest response, probing for further multi-dimensional learning from students. This process fosters internally motivated, goal-directed behavior to solve problems.

In order to improve the critical thinking ability of students in my gross anatomy courses, I demonstrate higher cortical models of learning when discussing and solving questions in clinical case studies. Students utilize critical thinking skills like skepticism, flexibility and creativity in a systematic manner when working with case studies: making observations, subsequent analysis of details, asking relevant questions for clarification, constructing hypotheses to secure the solution, and communicating, justifying and defending ideas to others while being receptive to alternate solutions[5-9].

This study also investigates whether the provision of diagnoses to case studies used in class has any effect on the sharpening of critical thinking skills. According to literature regarding diagnosis, inexperienced clinicians have a tendency to use a typical, biased pattern that leads to an initial answer, become "anchored" to this solution, and then neglect to find alternate causes to the case (opposing critical thinking practice)[10]. Did students without the diagnoses fall into the same trap and settle on the easiest solution available, without considering all the evidence and biases, when making a diagnosis? Will students provided with the diagnoses be skeptical of the answers, curious enough to explore the evidence (symptoms, history and presentation) to truly understand all facets of the case study and secure a solution on their own, or will they accept the given diagnosis as fact? Comparative statistics between semesters reveal whether an established solution to a case study will have any effect on critical thinking.

Materials and Methods

On the first day of the anatomy course (Human Gross Anatomy, students in the Occupational Therapy major; and Basic Gross Anatomy, students in Athletic Training and Pre-

Professional Biology majors), I had students complete a test (pre-test) consisting of nine anatomically-related questions that were of a hypothetical, developmental or behavioral nature (refer to *Scientific Evidence for Analysis of Questions*) in 25 minutes. On the last day of the course, students were given the same nine questions (post-test), time limit, and a new instruction: because evolutionary principles are stressed during the course, if students suspect an evolutionary purpose is involved in each answer, they are to elaborate on its purpose in their answer. Testing was completed in the fall of 2007 (*f1*, n = 34) to classes that had to determine the diagnoses to clinical case studies, and in the fall of 2008 (*f2*, n = 40) to classes that were provided with the diagnoses within the same case studies. After both sets of tests were collected for each semester, answers are evaluated for accuracy by using a scale based on the factual evidence available (Figure 1).

Figure 1. Scale for Responses to Pre-test and Post-test

1-5; numerical values for answers provided in pre-test and post-test for hypothetical (H), developmental (D) and behavioral (B) questions

1: student did not attempt to answer questions (nothing written)

2: student did not attempt a hypothesis for H question; student answered H, D and B questions incorrectly without support of factual evidence

3: student attempted a hypothesis for H question with some support of factual evidence; student answered D and B questions with some support of factual evidence

4: student attempted a hypothesis for H question with support of factual evidence but did not either include evolutionary reasoning or did not use evolutionary knowledge correctly; student answered D and B questions with support of factual evidence but did not either include evolutionary reasoning or did not use evolutionary knowledge correctly.

5: student attempted a hypothesis for H question with support of factual evidence which included correct usage of evolutionary reasoning; student answered D and B questions with support of factual evidence and included correct usage of evolutionary reasoning

Questions 1, 4, 5, 6, 8 and 9 are hypothetical, there are many hypotheses as to the function of the structure (resembling open-ended case studies, many solutions possible); questions 2 and 7 are developmental, factual evidence supports one globally-accepted answer (resembling closed case studies, one possible solution); and question 3 is behavioral. All of the answers were subjectively analyzed (refer to *Scientific Evidence for Analysis of Answers* below), given a numerical value from the scale, and collated (Tables 1 and 2). The numerical data was used to generate descriptive statistics and data compiled for answers ranked 4 and 5 (reflects most critical thinking, Figure 1) were used for comparative statistics (Tables 3, 4, 5 and 6).

Scientific Evidence for Analysis of Answers

Q1. What is the function of the cranial sinuses, mucosal lined cavities in the anterior frontal, maxillary, ethmoid, and sphenoid bones, (these are the sinuses that often become infected and lead to "sinusitis" or sinus headaches)?

Four hypotheses that have factual support: 1) the sinuses increase the surface area of mucosa, increasing heat and moisture in the air that passes through the nasal cavity (promoting easier gas exchange in the respiratory system); 2) the air-filled extensions decrease the weight of the skull (negligible difference); 3) distinguishes the shape of the adult face from the face of the new born (sinuses diminished in the infant skull); and 4) phonation or increasing resonance to the voice (bone conduction versus air conduction).

Q2. The extra set of molars known as the wisdom teeth do not have an apparent function in modern-day humans, often removed through surgery. Why are these teeth present?

The third set of molars that appear in the latter part of the second decade, hence called "wisdom teeth," can become impacted (lodged beneath) because of lack of room in the jaw. Mandible and maxillae in ancestral man were much larger in order to masticate unprepared food (raw food containing non-digestible structures such as shell, bone, and scales, that was not cut into smaller pieces), and could accommodate the extra set of teeth that aided in the mastication process. As the food preparation process evolved, and humans died less from food borne illness, the need for the larger jaw was not necessary, bones gradually became smaller and more refined.

Q3. Why is the act of smiling (bearing the canines and other teeth) in humans a friendly gesture, whereas, in the rest of the animal world bearing the canines/fangs is a threatening gesture, an act of aggression and warning? What is unique about the human species that may explain this non-verbal form of communication?

There are two behavioral theories: human babies are completely defenseless after birth, but the instinctual act of bearing the flesh-tearing teeth (which are not present at birth) is still exhibited to give an initial warning to the larger humans, older humans re-interpret the aggressive act as something positive, smiling, and an acceptable form of non-verbal communication throughout their social development; canines are smaller in humans than other carnivores and the bearing of canines is less of a threatening gesture and translated to a socially acceptable gesture.

Q4. The foramen lacerum, part of the passageway in which the internal carotid artery passes (superior to the foramen) supplies blood to the anterior 2/3 of the brain, is surrounded by jagged bone fragments (giving the lacerated appearance). It has been suggested that the foramen is gradually sealing itself and that is why the fragments appear as they do. What structure(s) might have passed through this foramen?

The jagged appearance of the foramen is actually only observed in the dry skull and in the living is enclosed by a cartilaginous shell that seals the jagged opening to prevent any damage to the internal carotid artery, passing horizontally and superiorly. A few minute blood vessels and nerves pass vertically through the foramen. Student-produced hypotheses include the following: as the anterior region of the human brain (pre-frontal cortex) developed more importance, the arterial supply for the lobes has increased in importance as well, and the need for a passageway that protects the artery more effectively (cartilage becoming bone); another larger vessel or nerve occupied the space that played a more important role in the brain of early man; and remnant/artifact of the skull of lesser complex species.

Q5. Why does a cerebral hemisphere receive sensory input from the opposite (contralateral) side of the body and why does a cerebral hemisphere project motor output to the contralateral muscles and glands in the facial region, trunk and limbs?

This relationship is largely unknown.

Q6. Why is the dorsal root ganglion, structures housing the cell bodies of sensory neurons projecting to the spinal cord, located externally to the vertebral canal in the vertebral column (clinically relevant for sensory dysfunction, i.e.herniated intervertebral discs)? Embryological development produces this organization, but why do the cell bodies of developing sensory neurons develop external to the spinal cord?

Because the receptors of spinal sensory neurons are present in tissues exposed to possibly harsh elements of the environment, it is not beneficial to have the cell bodies (control centers) of these neurons in the same tissues that are vulnerable to external forces. The sensory cell bodies are located in the dorsal root ganglion adjacent to the spinal column housed in the vulnerable bony intervertebral (IV) foramen. During embryological development, neural crest cells (precursors to sensory spinal neurons and other cells) develop external to the neural tube (precursor to the spinal cord). Because of this organization, the dorsal root ganglion can be impinged by soft tissues in the vertebral column, i.e. herniated IV disc leading to pain and dysfunction. A hypothesis discussed in class: organization of the many sensory inputs within the external dorsal root ganglion reduces the need for neurons to organize in the gray matter (dorsal horn), reducing the dorsal horn and allowing more room for the numerous sensory tracts in the white matter.

Q7. Why are there variations in skin color?Are their any advantages to each variation?

Variations in skin color types evolutionarily developed in relation to the degree of exposure and intensity of the sun's rays. For those people that have ancestral origin closer to the equator, where the earth receives the most direct rays from the sun, skin color became darker (more melanin produced and released in the dermis and epidermis) to absorb the sun's damaging ultraviolet (UV) rays and reduce destruction to the skin. For those people that have ancestral origins further away from the equator, the lighter colored skin (less melanin) has the ability to produce more vitamin D/cholesterol (critical for calcium absorption for strong bones and teeth) when exposed to the sun's energy, compared to darker skin. Because the people with lighter skin color had limited sun exposure, the lighter colored skin developed an adaptation to capture the limited sun's energy more efficiently.

Q8. Why do men typically lose hair on the top of the head as they age, while the hair in the eyebrows, nose and ears grows vigorously as they age?

Hair insulates the scalp and helps to retain a large percentage of body heat (like fur does for lower animals), necessary to maintain constant optimal body temperature for all metabolic processes, which would dissipate if the hair was not present. Because men have larger musculoskeletal builds comparatively, they generate more heat to maintain the larger mass. As men age, their metabolic rates decrease and subsequently they require less heat, accomplished by the dissipation of heat from the scalp. Di-hydrotestosterone (DHT), a converted form of testosterone that increases in concentration as men age, targets the hairs follicles on the scalp and decreases the longevity of this hair. This is the most accepted explanation for non-pathological hair loss on the head. Hair in the nose, ears and above the eyes helps to capture any foreign material that would affect the special senses. As to why this hair grows more vigorously as we age could be related to the decreased ability to regenerate damaged tissues as we get older. Less damage from foreign debris means less need for regeneration.

Q9. Why are there genetic variations that result in abilities that have no apparent advantage (tasting PTC, red-green color blindness, tongue curling)?

Common hypotheses included: functions derived from these genetic traits may have been very evident early in our evolution, according to the environment, and are obsolete in modern man; the genes could be linked to other genes of more importance for survival, and presence of these vestigial traits indicate presence of more beneficial genes; and for those genes that are expressed in the heterozygous phenotype may code for another function different from the homozygous phenotype and vice versa.

Results

The number of answers for each numerical value for each question in both tests was counted and used to determine the mean and SD for both semesters, fall 2007 (*f1*) and fall 2008 (*f2*) (Tables 1 and 2). Overall, descriptive statistics depict an increase in the means of the responses from pre-test to post-test (Table 1 and 2).

Table 1. Descriptive statistics* for pre-test, post-test data for fall 2007 (*f1*)

Questions	Test	1's	2's	3's	4's	5's	Mean	SD
Q1	Pre	4	15	10	3	1	2.33	0.85
	Post	8	2	12	8	3	2.88	1.29
Q2	Pre	6	8	13	5	1	2.60	1.05
	Post	3	3	15	7	5	3.24	1.12
Q3	Pre	9	7	11	6	0	2.42	1.09
	Post	0	1	22	8	2	3.33	0.65
Q4	Pre	19	11	3	0	0	1.51	0.67
	Post	15	4	5	4	5	2.39	1.54
Q5	Pre	16	16	0	1	0	1.58	0.66
	Post	11	11	10	1	0	2.03	0.88
Q6	Pre	11	13	5	4	0	2.06	0.99
	Post	2	15	7	8	1	2.73	1.01
Q7	Pre	1	1	21	7	3	3.30	0.81
	Post	12	4	10	6	1	2.39	1.25
Q8	Pre	15	9	8	1	0	1.85	0.91
	Post	12	4	10	7	0	2.36	1.19
Q9	Pre	14	8	9	0	2	2.03	1.13
	Post	10	6	6	5	6	2.73	1.50

* Generated via SPSS V. 15.0

For question 1, pre-test answers (*f1*: mean 2.33, SD 0.85; *f2*: mean 2.54, SD 0.72) include simplistic responses that reflect the most common hypotheses, and post-test responses (*f1*: mean 2.88, SD 1.29; *f2*: mean 3.25; SD 0.71) offered interpretations of function and evolution in man, respectively: mucosal-lined cavities provide extra moisture for structures in the head, preventing dessication and aiding the immune system (lymphatic drainage, filtering bacteria/viruses); and skulls in early man were heavier and sinuses were larger (greater weight difference). However, there were some novel hypotheses: sinuses were vestibular devices, increasing balance in lower animals which is needed for head orientation for hunting; and that the weight difference produced by sinuses is more significant in animals that require lighter skulls for speed (*f1*).

Table 2. Descriptive statistics* for pre-test (n=39), post-test (n=40) data for fall 2008 (*f2*)

Questions	Test	1's	2's	3's	4's	5's	Mean	SD
Q1	Pre	3	14	20	2	0	2.54	0.72
	Post	1	3	21	15	0	3.25	0.71
Q2	Pre	5	8	18	7	1	2.77	0.99
	Post	0	9	16	11	4	3.25	0.93
Q3	Pre	6	12	20	1	0	2.41	0.79
	Post	0	3	19	14	4	3.48	0.78
Q4	Pre	19	15	4	1	0	1.67	0.77
	Post	0	5	22	7	6	3.35	0.89
Q5	Pre	19	14	6	0	0	1.67	0.74
	Post	5	9	22	2	2	2.68	0.94
Q6	Pre	24	12	3	0	0	1.46	0.64
	Post	4	9	23	2	2	2.73	0.91
Q7	Pre	1	3	27	8	0	3.08	0.62
	Post	1	2	22	13	2	3.33	0.76
Q8	Pre	18	10	8	3	0	1.90	1.00
	Post	3	7	24	6	0	2.83	0.78
Q9	Pre	17	7	9	3	3	2.18	1.30
	Post	3	6	17	6	8	3.25	1.17

* Generated via SPSS V. 15.0

When asked the second question, pre-test answers (*f1*: mean 2.60, SD 1.05; *f2*: mean 2.77, SD 0.99) describe the function of wisdom teeth (vestigial set of 3rd molars) as replacements for lost/rotten molars, state some part of the accepted evolutionary theory, or report no function. The majority of post-test responses (*f1*: mean 3.24, SD 1.12; *f2*: mean 3.25, SD 0.93) re-state evolutionary reasoning, but there were 2 novel ideas: the teeth are left over from herbivore ancestors for grinding vegetation, alluding to our omnivorous digestion (*f1*); and early man ate food off the bone and the edible portion on long bones would be more effectively gnawed on by the deeper set teeth (*f2*).

When answering the third question on pre-tests (*f1*: mean 2.42, SD 1.09; *f2*: mean 2.41, SD 0.79), many students attempted to explain smiling as a positive gesture: because canines are smaller in humans they are not used for the purpose of threat/ aggression, and the gesture adopted another meaning; and humans have other gestures and language to translate aggression. Post-test answers (*f1*: mean 3.33, SD 0.65; *f2* mean: 3.48, SD 0.78) included new references to carnivores: large canines establishes dominance/alpha male or female in a pack for lower animals, whereas, humans have language and other means to express dominance in a group (*f1*); and humans are animals born without teeth, therefore, less prominence is placed on teeth as a visual warning (*f2*).

Pre-test statistics for question 4 (*f1*: mean 1.52, SD 0.67; *f2*: mean 1.67, SD 0.77) reflected unfamiliarity with the foramen lacerum. However, post -test responses (*f1*: mean 2.39, SD 1.54; *f2*: mean 3.35, SD 0.89) produced two distinctive hypotheses: the head was in a different position in quadruped ancestors and the muscles needed to support the head may have required another nerve or blood vessel supply, and as humans evolved, the need for these supportive muscles/nerve supply/blood vessels gradually disappeared (*f1*); and the novel idea that improper brain development *in utero* might have constricted a vessel in this foramen and lead to miscarriage/natural birth control (*f2*).

The cerebral hemisphere's relationship to the opposite side of the body, question 5, is largely unknown, and this was reflected in the pre-test data (*f1*: mean 1.58, SD 0.66; *f2*: mean 1.67, SD 0.74). However, two meritorious hypotheses appeared in the post-test data (*f1*: mean 2.03, SD 0.88; *f2*: mean 2.68, SD 0.94): 1) when early man was being attacked, the naturally dominant side of the body would be used to shield or attack the predator, but the hemisphere of the brain controlling the dominant side is on the opposite side of the cranium/head that is being exposed to the violence- the contralateral hemisphere for the dominant side of the body would be more protected than the side of the head facing the attacker (*f1* and *f2*); and 2) the projection of light rays from the peripheral visual field onto the nasal hemi-retina (nose shadow), forms a human's primary sensory contralateral projection system, all other neural projections followed this pattern (*f2*).

In question 6 of the pre-test (*f1*: mean 2.06, SD 0.99; *f2*: mean 1.46, SD 0.64), the majority of students state that there is more room for the sensory neurons to grow and develop, compared to the motor system, (flawed reasoning, motor axons grow freely outside the cord) (*f1* and *f2*). Post-test hypotheses (*f1*: mean 2.73, SD 1.01; *f2*: mean 2.73, SD 0.91), the most diverse in this study (*f1* and *f2*), best reflect the students' progression toward critical thinking and their burgeoning reasoning: 1) hypothesis regarding sensory spinal tracts requiring more room in the white matter; 2) compression of external sensory ganglion from pathology (i.e. cancer) or herniation of an IV disc

indicates damage that could eventually affect other regions of the vertebral column, pain indicates weakness, and guarding results; 3) cell bodies of sensory neurons outside the spinal cord are part of the PNS and have the ability to repair (regenerative sprouting) if damaged, whereas, motor neuron cell bodies in the spinal cord are part of the CNS and cannot repair.

Question 7, the most accurately answered question in the pre-test (*f1*: mean 3.30, SD 0.81; *f2*: mean 3.08, SD 0.62), was not expanded upon in the post-test (*f1*: mean 2.39, SD 1.25; *f2*: mean 3.33, SD 0.76). With regard to the lower post-test mean (*f1*), pre-test scores demonstrate that students already possessed knowledge about skin tone variation and may have not been challenged by the question.

The familiar phenomenon of hair loss in men in Question 8 include pre-test answers (*f1*: mean 1.85, SD 0.91; *f2*: mean 1.90, SD 1.00) pertaining to the increase of testosterone. Post-test responses (*f1*: mean 2.36, SD 1.19; *f2*: mean 2.83, SD 0.78) displayed a lack of critical thinking to actually link the loss of hair with other themes, such as evolution, and students, typically, did not address the growth of hair in the ears, eyebrows and nose as men age in either test.

Pre-test hypotheses of question 9 (*f1*: mean 2.03, SD 1.13; *f2*: mean 2.18, SD 1.30) and post-test responses (*f1*: mean 2.73, SD 1.50; *f2*: mean 3.25, SD 1.17) demonstrate a strong evolutionary genetics background: genes with no apparent function may have had more significance in ancestors or may have more importance later in our evolutionary development; mutations are necessary for survival of a species confronted with new environmental/geographical challenges, some mutations are artifacts of the process; and gene linkage between genes that encode apparent low function traits and genes that encode more vital functions, whether inheritance is recessive or dominant.

Comparative statistics calculated for the sum of 4 and 5 values reflect the most advanced critical thinking for this study (Figure 1). Responses are counted for each question for each test for each semester and placed into three categories (Tables 3 and 4): 1) total number of responses for all nine questions (Q1-Q9), overall effect; 2) responses for "hypothetical" questions Q1, Q4-6, Q 8-9; and 3) responses for "developmental" questions 2 and 7. To determine if there is a significant ($p < 0.05$) increase in the post-test results, chi-squared statistics and the *p* values (tables 3 and 4) are calculated using the formula below using the pre and post-test data (Table 3 and 4).

$$X^2 = \sum_{i=1}^{k} \frac{(Observed - Expected)^2}{Expected}$$

Table 3: Comparative statistics for sum of 4 and 5 values for pre-test, post-test (*f1*)

Categories	n	c^2 value	*p* value
1.Q1-Q9 (total)	111	16.65	$p < 0.001$
2.Q1, 4-6, 8-9 (hypothetical)	60	21.6	$p < 0.001$
3.Q2 and Q7 (developmental)	35	0.258	$p = 0.610$

Table 4: Comparative statistics for sum of 4and 5 values for pre-test, post-test (*f2*)

Categories	n	c^2 value	*p* value
1.Q1-Q9 (total)	133	42.29	$p< 0.0001$
2.Q1, 4-6, 8-9 (hypothetical)	68	28.47	$p< 0.0001$
3.Q2 and Q7 (developmental)	46	4.26	p = 0.039

Chi-squared results and the corresponding *p* values reveal a highly significant statistical increase between pre and post-tests for categories 1 and 2 for both semesters (*f1* = $p < 0.001$, *f2* = $p < 0.0001$); and the developmental questions of category 3 for *f2* was significant ($p = 0.039$) (tables 3 and 4). Statistically, the fall 2008 group showed the greatest improvement in post-test data. To determine if there was any significant difference between the data collected for the two semesters, comparative statistics were employed in two meaningful ways: the entire batch of values (4s and 5s) for both pre-test and post-test for each semester (table 5); and the same values for post-tests only for each semester (solely expressing the skills gained from the semester of instruction) (Table 6).

Table 5: Comparative statistics for sum of 4 and 5 values, (*f1*) and (*f2*)			
Categories	**n**	**c^2 value**	***p* value**
1.Q1-Q9 (total)	244	1.98	$p = 0.1594$
2.Q1, 4-6, 8-9 (hypothetical)	128	.50	$p = 0.4795$
3.Q2 and Q7 (developmental)	81	1.49	$p = 0.2222$

Table 6: Comparative statistics for 4 and 5 values, post-test only, (*f1*) and (*f2*)			
Categories	**n**	**c^2 value**	***p* value**
1.Q1-Q9 (total)	181	4.028	$p = 0.0448$
2.Q1, 4-6, 8-9 (hypothetical)	104	.6154	$p = 0.4328$
3.Q2 and Q7 (developmental)	49	2.469	$p = 0.1161$

Chi-squared results revealed that significance was achieved from the post-test results only when all questions (Q 1-9) were considered ($p = 0.0448$), (Table 6).

Conclusion

Based on descriptive and highly significant comparative statistics (post-test results), gross anatomy students demonstrated an increased ability to answer anatomically-related questions by using factual evidence, evolutionary principles and improved critical thinking skills (gained from case study analysis), especially when formulating hypotheses to those questions that do not have universally-accepted solutions. When examining both descriptive and comparative statistics between fall 2007 and fall 2008, it was evident that there was a significant increase in critical thinking aptitude in the fall 2008 students, the group that was given the diagnoses when solving case studies. Skepticism, an important component of critical thinking, may have influenced these results: the fall 2008 group are able to determine their own solutions to the case studies and compare them to the answers provided. Perhaps, this process of comparison allowed the fall 2008 students the unique opportunity to increase their proficiency with critical thinking skills and this was reflected in the results of the post-tests. Based on this data, I will continue to use case studies including diagnoses to improve critical thinking in my students and I look forward to conducting similar research on higher cortical processing in other courses.

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Identification of Dopamine D2 Receptors in Gill of *Crassostrea virginica*

by

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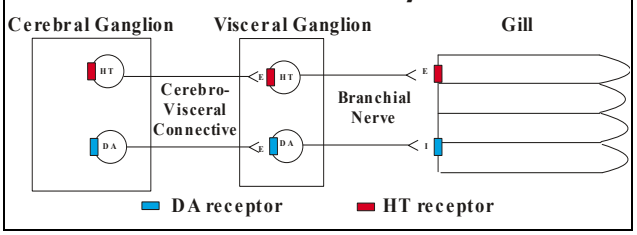
Abstract

The lateral epithelial cells of gill of *Crassostrea virginica* are innervated by dopamine and serotonin nerves that regulate the beating rate of their lateral cilia. Terminal release of dopamine slows down the beating rate of the cilia, while serotonin release increases the beating rate. Previously, we showed that the dopaminergic, but not the serotonergic, mechanism regulating the beating rate of the lateral cilia was disrupted by manganese treatments and that this disruption was occurring postsynaptically, at the level of the dopamine receptor or further downstream in the signal transduction pathway. In humans manganese toxicity causes Manganism, a neurological disorder with clinical symptoms similar to Parkinson s disease. In this study we utilized pharmacological agents and an immunohistofluorescence technique to characterize the dopamine receptor type present on the lateral ciliated cells of *C. virginica* gill. Agonists and antagonists to dopamine D1 or dopamine D2 receptors were applied to gill sections and beating rates of the lateral cilia were measured by stroboscopic microscopy. The D2 agonists and D2 antagonists were effective in mimicking or blocking, respectively, the inhibitory actions of dopamine on lateral cilia beating, while application of either D1 agonists or D1 antagonists had no significant effect. In other experiments we used an epilume fluorescence microscopic fitted with FITC filters to view gill sections treated with a primary antibody against D2 receptors and a FITC-linked secondary antibody. Control gill sections without primary antibody exposure were similarly treated and viewed. The D2 antibody treated sections showed bright fluorescent receptor-antibody complexes present at the lateral ciliated cells and other areas of gill, when compared to controls. The results of our immunofluorescence study identify the presence of D2-like receptors on the lateral ciliated cells of *C. virginica* gill and our pharmacological results indicate that D2-like receptors are the postsynaptic dopamine receptors involved in the cilio-inhibitory response of the lateral cilia. The results of this study, when combined with our previous work, further suggest that the mechanism of action that underlies the dopaminergic neurotoxicity of manganese in gill of *C. virginica* involves disruption of D2-like receptors. *C. virginica* continues to provide a simple yet good model with which to study the physiology of dopaminergic systems as well as the pharmacology of drugs affecting biogenic amines.

Introduction

The Eastern Oyster, *Crassostrea virginica*, has a reciprocal dopaminergic and serotonergic innervation of the lateral ciliated cells of gill epithelia, similar to that of *Mytilus edulis*[1]. The innervation originates in the cerebral and visceral ganglia, and controls each gill via a branchial nerve. Excitation of the dopaminergic circuit results in a terminal release of dopamine at the level of the gill epithelium that slows down and stops the beating of the cilia of the lateral cells. Excitation of the serotonergic circuit results in a terminal release of serotonin (5-hydroxytryptamine) that speeds up the beating of the cilia of the lateral cells (Fig. 1). Since the animal's nervous system activity is directly related to a measurable physiological response, the ganglia and gill preparations of *C. virginica* can serve as a useful model with which to study dopaminergic and serotonergic systems and the chemicals that affect the release and activity of these biogenic amines.

Fig. 1. Schematic of Gill Lateral Cell Innervation



Manganese is a required trace element that serves as an enzyme cofactor or activator for numerous reactions of metabolism[2]. While essential in trace amounts, excessive manganese exposure can result in toxic accumulations in the human brain causing extrapyramidal symptoms similar to those seen in patients with idiopathic Parkinson's Disease[3-7], a dopaminergic degenerative disease of the substantia nigra pars compacta. Manganese-induced Parkinsonism was first described in 1837 in two manganese ore-crushing mill workers[8] and has since been referred to as Manganism[3,9-11]. Although Manganism has been recognized for some time, the primary mechanism underlying manganese neurotoxicity remains elusive.

Previously we determined that *C. virginica* readily accumulates manganese into its ganglia and tissues[12] and that elevated tissue manganese was associated with a reduction of dopamine in the oyster's cerebral ganglia, visceral ganglia and gill, while having no effect on levels of other biogenic amines, including serotonin, norepinephrine and octopamine[13]. Using oyster ganglia and gill preparations, we also showed that the dopaminergic, but not the serotonergic, mechanism regulating the beating rates of the lateral cilia was disrupted by manganese. Oysters pre-treated for 3 days in 50 - 500 μM manganese had an impaired cilio-inhibitory response to exogenous dopamine whether applied to the cerebral ganglia, the visceral ganglia or directly to the gill[14,15]. These results suggested, at least at the level of the gill, that manganese disruption of the dopaminergic response was occurring postsynaptically either at the dopamine receptor or further downstream in the signal transduction pathway.

Dopamine receptors are metabotropic G protein-coupled receptors that indirectly control the opening and closing of ion channels located at other sites on the post-synaptic membrane. They are classified as either D1-like or D2-like and each family can be divided into various subtypes[16-18]. Dopamine D1-like receptors include human subtypes D1 and D5, and are coupled to G protein $G\alpha_{s,}$ which subsequently activates adenylyl cyclase raising levels of cAMP. Dopamine D2-like receptors include human subtypes D2, D3 and D4, and are coupled to the G protein $G\alpha_{i,}$ which directly inhibits adenylyl cyclase lowering levels of cAMP. A detailed review of the physiology, signaling and pharmacology of dopamine receptors was recently published by Beaulieu and Gainetdinov[19].

In this study we sought to characterize the dopamine receptor type present on the lateral epithelial cells of gill of *C. virginica* to gain more insight into the mechanism of action underlying manganese neurotoxicity.

Materials and Methods

Adult *C. virginica* of approximately 80 mm shell length were obtained from Frank M. Flower and Sons Oyster Farm in Oyster Bay, NY, USA. Oysters were maintained in the lab for up to two weeks in temperature-regulated aquaria in Instant Ocean artificial seawater (ASW) obtained from Aquarium Systems Inc. (Mentor, OH, USA) at 16 - 18ºC, specific gravity of 1.024 ± 0.001, salinity of 31.9 ppt and pH of 7.8 ± 0.2. Each animal was tested for health prior to experimentation by the resistance it offered to being opened. Only animals that fully closed in response to tactile stimulation and required at least moderate hand pressure to being opened were used for the experiments.

A68930 ([1R, 3S] 1-aminomethyl-5,6-dihydroxy-3-phenylisochroman HCl), B-HT 920 (6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]azepine), clororprothixene (2-Chloro-9-(3-dimethylamino-propylidene)-thioxanthene hydrochloride), dopamine hydrochloride, ergocryptine (2-Bromo-α-ergocryptine methane-sulfonate), ergonovine malate, piribedil (2-[4-(1,3-Benzodioxol-5-ylmethyl)-1-piperazinyl]pyrimidine maleate), propylpiperidine (R(+)-3-(3-Hydroxyphenyl)-N-propylpiperidine hydrochloride, serotonin hydrochloride, and SKF-89145 hydrobromide (4-(3,4-Dihydroxyphenyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Just prior to use, dopamine and the various D1-like or D2-like agonists and antagonists, were dissolved in ASW containing 10 mg% ascorbic acid buffered to pH 7.2 with sodium bicarbonate (ASWA) to retard oxidation, as described by Malanga[20]. Primary and FITC-linked secondary antibodies used for the immunohistofluorescence receptor staining were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were obtained from Fisher Scientific (Suwanee, GA, USA) and were of analytical grade or higher.

Pharmacological Action of D1-like and D2-like Agonists and Antagonists on Beating Rates of the Lateral Cilia

In order to observe the effect of dopamine and the various dopamine D1-like or D2-like agonists and antagonists on the beating rate of the lateral cilia, gills were positioned in observation chambers and the cilia of the medial gill lamina were viewed at 100 - 200x magnification through an Olympus CK inverted microscope with transmitted stroboscopic light from a Grass Instruments PS 22 Photo Stimulator. The cilia of the lateral epithelial cells beat in a metachronal wave pattern[21]. This wave pattern allows for the beating rates of the cilia to be measured by synchronization with stroboscopic light[22]. When the flashing rate of the strobe is synchronized with the beating rate of the cilia, the lateral cilia appear motionless in a characteristic horseshoe like configuration. At all multiple synchronizing rates above the one corresponding to the true beating frequency, the wavelength of the beating cilia will appear to be a fraction of the true wavelength.

Since the lateral cilia tend to be quiescent at the start of the experiments, gills were first bathed 10 min in 10^{-5}M serotonin to activate the cilia and generate a steady cilia beating rate of 15+ beats/s prior to application of dopamine or the D1-like or D2-like agonists and antagonists.

A dose response for each D1-like and D2-like agonist was determined by measuring its effectiveness at reducing the beating rate of the lateral cilia, compared to dopamine. For each D1-like and D2-like antagonist, a dose response of dopamine against 10^{-4}M antagonist was determined by measuring the antagonist's effectiveness at blocking the cilio-inhibitory effect of added dopamine. Statistical analysis for each agonist or antagonist trial was determined by nonlinear regression analysis.

Immunohistofluorescence Staining of Dopamine Receptors

Gills were excised from oysters and fixed with 4% paraformaldehyde, 25 mM Hepes and 0.1% Triton X100 for 10 min, washed with PBS-Triton 3 times for 10 min, immersed in a blocker (bovine serum albumin) for 45 min, and then washed again 3 times with PBS-Triton. The gill were then immersed into primary dopamine D2 receptor antibody (1:500 dilution) overnight at 4ºC then washed 3 times with PBS-Triton. The gill were visualized with FITC secondary antibody (1:200 dilution) for 1 hour at room temperature in the dark, then washed 3 times with PBS-Triton. The tissue were dehydrated in an alcohol series to xylene, then vacuum embedded in paraffin. Sections were cut at 10 micron and mounted on slides. A warming plate was used to flatten the sections, which were then cleared with xylene and rehydrated in an alcohol series to water. The sections were mounted in 10% sodium borate, 3% n-Propyl gallate in glycerol and viewed with a Zeiss epilume fluorescence microscope fitted with FITC filters (Ex 470 nm, Em 525 nm). Photomicrography was done with a ProgRes C3 Peltier cooled camera. Control gill sections were similarly treated without the primary antibody.

Results

Adding dopamine (10^{-6} - 10^{-3}M) to isolated gill caused a dose dependent decrease in beating rates (Fig. 2). The D2 agonists propylpiperidine and ergocryptine were as effective as dopamine in activating dopamine postsynaptic receptors and slowing cilia. BHT 920 slowed the cilia, but was not as potent as dopamine, being about 1 log unit less effective (Fig. 2). The D1 agonists A768930 and SKF-89630, and the D3 agonist piribedil, were much less effective in mimicking dopamine at low concentrations (10^{-6} - 10^{-4}M, but did slow the cilia at the high dose of 10^{-3}M (Fig 3). The D2 antagonists chlorprothixene and ergonovine were effective in blocking dopamine from activating postsynaptic receptors and slowing cilia (Fig. 4), while the D1 antagonists droperidol was statistically ineffective at all dopamine concentrations in blocking dopamine, even at the dopamine concentration of 10^{-3}M (Fig. 5).

Because the pharmacological studies indicated that the postsynaptic dopamine receptors mediating slowing of cilia of the lateral cells are D2 type receptors, we used an FITC based technique to determine if postsynaptic dopamine D2 receptors were present in the lateral cells of the gill. The staining showed bright green fluorescence within lateral gill cells as well as other areas of the gill, confirming D2 receptors (Fig. 6).

Discussion

The study shows the postsynaptic dopamine receptors involved in the cilio-inhibitory response of the lateral cells of the gill are of the D2-type. The D1-type agonists and antagonists were not effective in mimicing or blocking, respectively, the

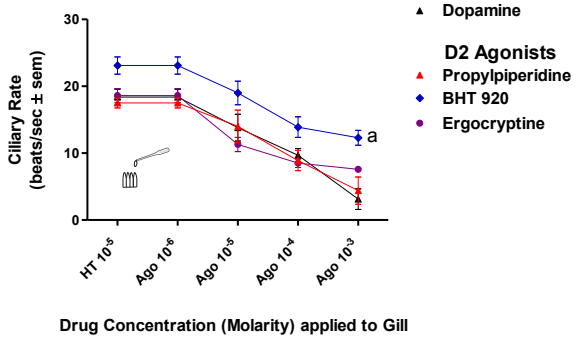


Fig. 2. Effects of dopamine and D2 agonists (ago) on the ablity to change beating rates (beats/sec) of lateral gill cilia when applied directly to gill. The gill was activated with 10^{-5}M serotonin. The D2 agonists were effective in slowing cilia. N=5 for each experiment. Statistical analysis was determined by nonlinear regression analysis, $^{a}p<0.001$ for comparison of dose response to that of dopamine.

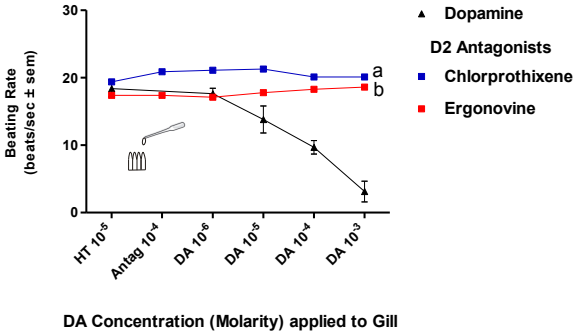


Fig. 4. Effects of the D2 antagonists (antag) (10^{-4}M) on the ablity to block the effects of dopamine on slowing beating rates (beats/sec) of lateral gill cilia when applied directly to gill. The gill was activated with 10^{-5}M serotonin. N=5 for each experiment. Statistical analysis was determined by nonlinear regression analysis, $^{a}p<0.001$, $^{b}p<0.05$ for comparison of dose response to that of dopamine.

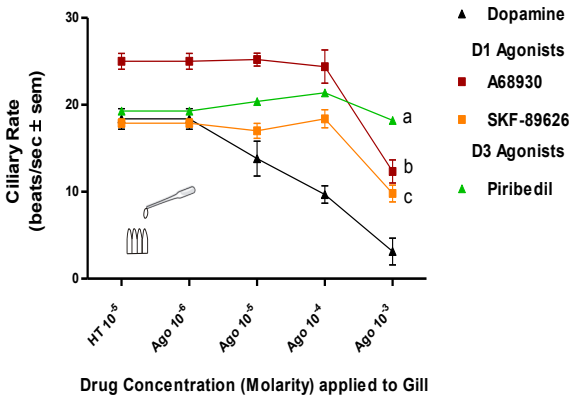


Fig. 3. Effects of D1 and D3 agonists (ag0) on the ablity to change beating rates (beats/sec) of lateral gill cilia when applied directly to gill. The gill was activated with 10^{-5}M serotonin (HT). The D1 and D3 agonists were not as effective as dopamine in slowing cilia. N=5 for each experiment. Drugs were significantly different from the dopamine response. Statistical analysis was determined by nonlinear regression analysis, $^{a}p<0.0001$, $^{b}p<0.001$, $^{c}p<0.05$ for comparison of dose response to that of dopamine.

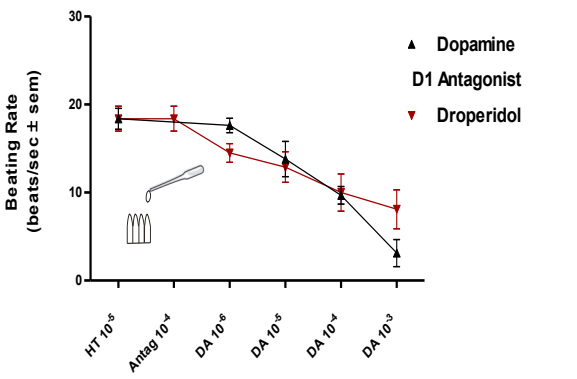


Fig. 5. Effects of the D1 antagonist (antag), droperidol (10^{-4}M), on the ablity to block the effects of dopamine on slowing beating rates (beats/sec) of lateral gill cilia when applied directly to gill. The gill was activated with 10^{-5}M serotonin. Statistical analysis was determined by nonlinear regression analysis. Droperidol was not effective in blocking dopamine. N=5 for each experiment

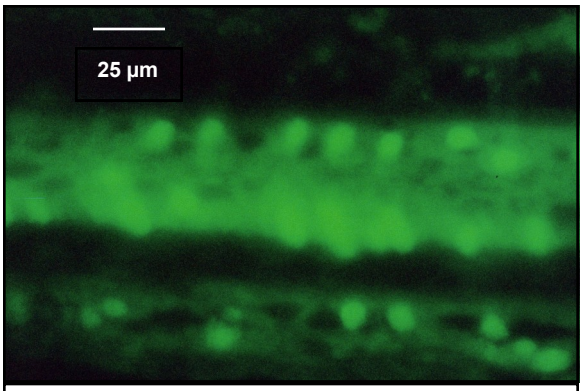


Fig. 6. Photomicrograph of gill treated with D2 and FITC antibodies.

actions of dopamine. The immunohistofluorescence staining verified that D2-type receptors were present in the lateral cells and other areas of the gill. Although manganese-induced Parkinsonism has been recognized for some time, the primary mechanism underlying manganese neurotoxicity remains elusive. Evidence from nonhuman primate data suggests a manganese-induced post-synaptic decrease of D2 -like dopamine receptor levels[23-25] may be partly responsible.

The actions of dopamine in bivalve molluscs is well documented. In most species it slows down the beating rate of the cilia of the gill lateral cells[26], but speeds up the beating rates of the frontal cells[27]. It causes contraction of the musculature of the gill[28], as well as contraction of the foot in *M. edulis*[29]. Dopamine stimulates adenylyl cyclase in pedal ganglia of *M. edulis*[30], as well as increasing cAMP in the gonads[31]. Stimulation of adenylyl cyclase suggests involvement of D1-like dopamine receptors, while D2-like dopamine receptors inhibit adenvlyl cyclase activity. The various effects of dopamine on different physiological and biochemical actions indicate that they may be mediated by different receptor types, each specific for a specific function. D1, D2, D3 and D4 receptors are present in the gastropod mollusk, *Aplysia californica*. The specific binding for each subtype varies differentially with age of the animal[32].

The present study shows that the gill and gill ganglia preparations are good models for pharmacological studies of dopamine function as well as the pharmacology of drugs affecting biogenic amines in nervous systems.

Acknowledgments

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