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MONTCLAIR STATE UNIVERSITY TO HOST THE MACUB CONFERENCE SATURDAY, NOVEMBER 1, 2008

DEAN HAMER AND DONALD PFAFF TO PRESENT KEYNOTE ADDRESSES AT 41st ANNUAL MACUB CONFERENCE

Dr. Dean Hamer

Dr. Hamer was born in Montclair, N.J. He received his B.A. from Trinity College, Connecticut and his Ph.D. from Harvard Medical School. He has worked at the National Institutes of Health for 24 years, where he is currently the Chief of the Section on Gene Structure and Regulation in the Laboratory of Biochemistry of the National Cancer Institute. Dr. Hamer's research has led to contributions in a variety of areas including recombinant DNA, drug and vaccine production, and gene regulation. He was a coinventor of animal cell gene transfer, and recently has begun a program on molecular therapeutics for HIV/AIDS. For the past nine years, Dr. Hamer has studied the role of inheritance in human behavior, personality traits, and cancer risk-related behaviors such as cigarette smoking. His discovery of genetic links to sexual orientation and the temperamental traits of sensation seeking and anxiety have changed the way we think about human behavior and raise a host of important scientific, social and ethical issues.

Dr. Hamer has published over 100 scientific papers and holds three patents in the biotechnology area. He is the author of "The God Gene: How Faith is Hardwired into our Genes". His book, “The Science of Desire”, co-authored with journalist Peter Copeland, has won widespread critical acclaim and was a 1994 New York Times "Notable Book of the Year". Their new book, “Living With Our Genes,” is a science best seller. Dr. Hamer's research has been described in Discover Magazine and other national publications.

Dr. Donald Pfaff

Dr. Pfaff will present the keynote address, “On The Status of Modern Neuroscience”. He received the A.B. degree magna cum laude from Harvard College in 1961 and a Ph.D. from the Massachusetts Institute of Technology in 1965. He held a National Merit Scholarship, Harvard National Scholarship, Woodrow Wilson Fellowship, MIT President's Award Fellowship, National Institutes of Health Predoctoral Fellowship and National Science Foundation Postdoctoral Fellowship.

Dr. Pfaff joined The Rockefeller University in 1966. Presently, he is a professor and head of the Laboratory of Neurobiology and Behavior at The Rockefeller University, is a brain scientist who uses neuroanatomical, neurochemical and neurophysiological methods to study the cellular mechanisms by which the brain controls behavior. Dr. Pfaff is the author of "Estrogens and Brain Function" (Springer, 1980); "Drive: Neurobiological and Molecular Mechanisms of Sexual Motivation" (MIT Press, 1999); and "Brain Arousal and Information Theory" (Harvard University Press, 2005). He has edited 'The Physiological Bases of Motivation' (1982), 'Ethical Questions in Brain and Behavior' (1984), 'Genetic Influences on the Nervous System' (CRC Press, 1999) and 'Hormones, Brain and Behavior' (5 volumes, Academic Press, 2002). He also is on the editorial boards of several scientific journals.

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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.

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DNA Identification: Can DNA Be Used For Authentication

by

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Introduction

This article examines the science of DNA identification and its possible use for authentication to provide security for On-line transactions. DNA (deoxyribonucleic acid) was discovered in the 1800's, but its role as the material of heredity was not interpreted for another fifty years thereafter. It is a genetic material unique to each individual and forensic scientists can use DNA in blood, semen, skin, saliva or hair at a crime scene, for example to identify a perpetrator. Understanding DNA has facilitated genetic engineering, the genetic manipulation of various organisms; allowing for genetic fingerprinting, or more accurately, DNA profiling. In DNA profiling, the lengths of variable sections of repetitive DNA, such as short tandem repeats and minisatellites, are compared between people. This method is usually an extremely reliable technique for identifying a criminal[1]. However, identification can be complicated if the scene is contaminated with DNA from several people[2]. DNA profiling was developed in 1984 by British geneticist Sir Alec Jeffreys[3] and first used in forensic science to convict Colin Pitchfork in the 1988 Enderby murders case[4]. People convicted of certain types of crimes may be required to provide a sample of DNA for a database. This has helped investigators solve old cases where only a DNA sample was obtained from the scene. DNA profiling can also be used to identify victims if mass casualty incidents such as 9/11[5].

By contrast, however, DNA analysis allows identification by reference to the information contained in any human cell nuclei, irrespective of which part of the body the cell comes from. The DNA in a human cell is unique. It is the product of sexual reproduction that combines half of the mother's DNA and half of the father's DNA. Every cell in an individual's body is the result of the cellular division, which copies the DNA in the newly fertilized cell into every other cell. As a result, DNA in a cell is identical throughout a human body but variable between any two humans, making it a natural alternative to artificial human identifiers, such as names or social security numbers. The notable exception is identical twins, who develop from a single fertilized cell and hence have identical nuclear DNA[6].

Authentication on the other hand, is the process requiring the subject to provide verification that he/she is who he/she claims to be. The most common method of identification is the use of a user-ID and password comparison. In a computer network, authentication must be handled carefully and correctly because not just people but processes, servers, and services associated with people must be authenticated as well[7,8].

Passwords are the most common form of authentication used in network security. It is not unusual for users to have multiple accounts, both debit and credit cards, on several systems, each requiring different PIN numbers. Properly used, password authentication is a good tool for controlling access to accounts and systems[9,10,11]. Due to the financial reward and because passwords are so widely used for access control, hackers have developed many ingenious ways of attacking the password files stored on database systems.

Another method of authentication commonly used to identify a subject is the use of a token, often a smart card. It is used primarily to confirm the identity of the user by carrying out a challenge/ response activity within the card. Because of its electronic nature, it either generates a value in response to an input data or it generates a time-sensitive value.

The new method employed to enforce authentication is the implementation of a biometrics-based authentication system. Biometrics authentication is mainly used by large security-conscious entities such as the banking and other related financial institutions for regulating access to sensitive information. In a recent published announcement however, the FBI wants palm prints, eye scans and tattoo mapping. According to the published report, the

FBI is gearing up to create a massive computer database of people's physical characteristics, awarding a $1 billion, 10-year contract to help create the database that will compile an array of biometric information from palm prints to eye scans. The FBI already has 55 million sets of fingerprints on file and has begun collecting images which will eventually be used as an additional means of making identifications. All being part of an effort to better identify criminals and terrorists[12].

The strength of most authentication systems lies in their strong cryptographic protocol which makes them almost impossible to compromise. Additionally, biometric systems are the most expensive authentication system to implement due to its complexities. For now, biometric authentication appears to be the solution to the problem of authentication in cyberspace.

Using DNA in Identification

Until recently, law enforcement officials, who over the course of many years have looked for things that uniquely identify individuals, quickly realized the DNA identification would be especially useful in legal cases, in the criminal courts. Since 1989, 14 convicted murderers in the United States owe their freedom and in death penalty cases their lives to the role DNA played in overturning their sentences. Faulty eyewitness accounts is one of the principal grounds for overturning prison sentences.

Like the fingerprints that came into use by the law-enforcement labs in the 1930s, each individual has a unique DNA fingerprint. Unlike the conventional fingerprint that occurs on the fingertips and can be altered due to an injury or by surgery, a DNA fingerprint is the same for every cell, tissue, and organ of an individual. So far, it cannot be altered in any form or shape by any known treatment. Consequently, DNA fingerprinting is rapidly becoming the primary method for identifying and distinguishing among individual human beings.

DNA fingerprints are useful in several applications of human healthcare research as well as in the justice system. Currently there are on-line fingerprint databases across the country shared and used by law enforcement agents. However, for many crimes no fingerprints are left behind. For example, for property related crimes it is possible to find and lift fingerprints from the scene of the crime but for other violent crimes such as rape or murder it's harder sometimes to find fingerprints.

DNA gives detailed results just as a fingerprint. In the forensic application commonly done today information is gleaned from regions that have spelling differences that are due to repetitions of some sequence. By taking that DNA and cutting it with an enzyme that recognizes a distinctive site, running it out by electrophoresis to be able to separate these fragments by size, and probing it with a piece if radioactive DNA from this region, one can visualize bands corresponding to the lengths of these fragments. Hence, each of these different chromosome configurations, each of these different spellings due to different number of repeats, can be visualized as different-sized bands on a ladder, much like a bar code.

These are some of the basic ideas underlying DNA fingerprinting, as it's popularly known or DNA identification. Within five years of the concept of DNA spelling differences being used for medical purposes, there were various private companies, such as Lifecodes, Selmae and others which started to provide DNA fingerprinting services to law enforcement officials. By 1989 the FBI had its own DNA fingerprinting laboratory in the Hoover building in Washington.

Increasingly in rape cases there is no need for a victim to testify about whether a sexual act took place. The DNA from a semen sample can be used to link a suspect to that semen sample. Some of these cases were dues to the successful work of the *Innocence Project*, founded by Barry Scheck and Peter Nuefeld pioneers in the use of DNA evidence in criminal cases[13].

Nearly 200 U.S convictions for other crimes have been overturned using DNA evidence. The average amount of time the exonerated spent in prison was 12 years. They come from 31 of the 50 U.S states. Even the FBI concur that of the many test results that otherwise they could never exclude with standard blood markers nearly a third of those people are exonerated upon DNA testing.

DNA and Computation

DNA was first used in computing to solve a small version of the directed Hamiltonian path Problem, an NP-complete problem[14]. DNA computing is advantageous over electronic computers in power use, space use and efficiency, due to its ability to compute in a highly parallel fashion. A number of other problems including simulation of various *abstract machines*, the *boolean satisfiability* problem, and the bounded version of the *traveling salesman* problem, have since been analyzed using DNA computing[15]. Due to its compactness, DNA also has a theoretical

role in cryptography where in particular it allows unbreakable one-time pads to be efficiently constructed and used[16].

Making DNA Fingerprints[17]

DNA fingerprinting is a laboratory procedure that requires five steps:

1. Isolation of DNA

DNA has to be recovered from the cells or tissues of the body. Only a small amount of tissue – like blood, hair, or skin is needed. The amount of DNA found at the root of one hair is usually sufficient.

2. Cutting, Sizing, and Sorting

Special enzymes called restriction enzymes are used to cut the DNA at specific places. The DNA pieces are sorted according to size by a sieving technique called electrophoresis. The DNA pieces are passed through a gel made from seaweed agarose. This technique is the biotechnology equivalent of screening sand through progressively finer mesh screens to determine particle sizes.

3. Transfer of DNA to Nylon

The distribution of DNA pieces is transferred to a nylon sheet by placing the sheet on the gel and soaking them overnight.

4. Probing

Adding radioactive or colored probes to the nylon sheet produces a pattern called the DNA fingerprint. Each probe typically sticks in only one or two specific places on the nylon sheet.

5. DNA fingerprint

The final DNA fingerprint is built by using several probes (5-10 or more) simultaneously. It resembles the bar codes used by grocery store scanners.

Can DNA be used for Authentication?

Based on modern comparative techniques which compares only a small set of features of the "non-coding" DNA it is possible to use DNA for authentication. Such sets of features also known as DNA profiles can be represented as an ordered set of numbers. The possibility of quantifying the DNA profiles represents a further advantage over other unique human features such as identification of an individual based on their entire body or of localized special bodily characteristics such as blood group or fingerprints as it allows for automated analysis. The only pre-condition is that the features comprised in a DNA profile must be sufficiently variable throughout the population to have an acceptable statistical likelihood that the profile is unique in that population, but also sufficiently regular to be responsive to cheap and efficient mass analysis.

Contemporary profiling techniques are increasingly automated but the elimination of artifacts of the profiling process requires careful judgments by properly trained scientists[18,19]. Future developments may allow initial profiling to be done by non-technicians outside of the laboratory[20]. An attempted comparison ("matching") of two DNA profiles in order to determine whether they are related will yield one of the three possible results (Table 1)[21].

The most important method of mass comparison is through the use of databases of DNA profiles from know persons each of which can be easily compared with every crime profile as in criminal cases, or such as those collected from credit card and loan applicants. Additionally, samples can be drawn from volunteers, some crime suspects and certain categories of offenders as it's currently being done by law enforcement officers from various jurisdictions. With common protocols different databases can be linked to expand the group of known persons whose profiles are regularly screened against crime scene sample[22]. This is the idea behind the establishment of national database such as Automated Fingerprint Identification System (AFIS) and Combined DNA Index System (CODIS) used by the enterprises, banks, hospitals retail stores and the government (FBI and other law enforcement agencies across the country).

The growing role of e-Commerce is changing the way we do business. Anyone with access to a computer, an Internet connection and a means to pay for purchased goods or services online can participate in e-Commerce. Most online consumers use credit or debit cards to pay for online transactions, but payment methods like "e-wallets" and debit cards are also becoming more popular. This electronic mode of payments is also providing opportunities for electronic fraud and the continued rise in online commerce is forcing consumers and businesses to focus on Internet security.

Table 1. Possible Outcomes of DNA Matching

Outcome	Description	Explanation/Interpretation
Null result	Profile comparison	This occurs when one or both samples are of insufficient quantity or quality (e.g. because of contamination by DNA from microbes) to yield an adequate DNA profile
Negative results (exclusion)	Profiles are different, no DNA match	This is conclusive that the two samples are derived from different individuals
Positive results (inclusion)	Profiles are the same, DNA match	This is evidence that the two samples are derived from the same human being or identical twins. Note that there are several important alternative hypotheses for a positive result

Source: Jeremy Gans and Gregor Urbas: DNA Identification in the Criminal Justice System – No.226. Australian Institute of Criminology, Canberra ACT 2601, Australia.

Table 2. Main Hypothesis for a DNA Match

Scenario	Description	Likelihood	Ways to Reduce Error
Coincidence	Crime sample comes from an unrelated person with the same DNA	A slight possibility for contemporary profiling methods. The possibility can be estimated by sampling profiles from the population of possible offenders. The statistical risk is increased if both suspect and offender come a genetically isolated group or if the suspect was located through a database search.	Increase the number of DNA features profiled. Test or exclude possible suspects, for example, by mass screenings or database searches.
Kinship	Crime sample comes from a related person with the same DNA profile	A higher possibility than the coincidence scenario if the suspect and perpetrator are first cousins or closer. If the suspect and perpetrator are identical twins then the likelihood is 100 per cent. Risk can be estimated using straightforward population genetics.	Increase the number of DNA features profiled. Test or exclude close relatives, especially identical twins.
Contamination	Crime sample is contaminated by DNA from suspect sample	A possibility if the suspect sample has never been near the crime sample. The risk of a false inclusion is higher where the profiling process replicates small amounts of DNA.	Separate profiling of suspect and crime samples. Introduce stringent crime scene and laboratory protocols to avoid contamination. Preserve part of the crime sample before testing (not possible for some crime samples).
False exclusions, which can occur through errors in the handling of samples or the reporting of result, are of less concern as they can be largely avoided through improved protocols.			

Since online transaction requires making payment at the time of purchase a consumer being concealed in anonymity can cheat by presenting stolen credit card for payment and a seller can cheat by overcharging or by taking payment and not delivering the merchandize already paid for. Additionally, a fraudster can set up "phantom" shop online to simply collect unsuspecting customer's payment in the form of credit card number(s) and vanish. Anonymity is a major problem plaguing online transactions. Although efforts are continuing in the development of stronger encryption, encryption alone cannot protect against hackers who have developed many ways of attacking the password files stored on database systems.

Conclusion

While DNA identification shows promise of unlimited use it is important to recognize that not all inclusions or exclusions will be of value in an investigation. The utility of DNA identification is entirely dependent on the correct characterization of crime scene samples, which may be compromised by poor crime scene management or deliberate misconduct. A persistent concern is the possibility of false inclusion, that is, matching profiles from samples with different human sources. There are several alternative hypotheses for a positive DNA match that must be considered[23] (Table 2[21]).

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In vitro Maturation Of NK Cells In The Human Umbilical Cord Blood Treated With Wheat Grass Extract: A Pilot Study

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ABSTRACT

The immuno-modulating activity of natural substances was evaluated to assess their effects on human umbilical cord blood mononuclear cells (MNC). Most of the MNC might have been immunologically primed as they are exposed to immunological stressors, while MNC from the human umbilical cord blood (hUCB) are not. During pregnancy, MNC of hUCB are isolated from bio-stressors by virtue of the trophoblast stratum between the placenta and its neighboring maternal endometrium. Hence, the MNC are well protected from bio-stresses. The trophoblastic layer prevents MNCs of the hUBC from being affected by various factors circulating in the maternal blood, so that the exposure of MNC to those factors is reduced. After treatment with eleven different natural substances, including five herbal extracts and six pure compounds, the immunophenotypic expression of MNC subsets was analyzed via fluorescence activated cell sorting (FACS) analysis. The data indicated that the extract of wheat grass (*Triticum aestivum* L.) intensified the population of $CD56^+$ natural-killer (NK) cells, whereas the propagation of other MNC was insignificant. NK cells separated with the positive magnetic bead cell isolation method (MACS) and treated with wheat grass extract showed marked cytotoxicity affecting the cells of Modal karyotype of human leukemia cell line, K562. This pilot study shows that the wheat grass extract likely promotes the *in vitro* maturation of NK cells in the hUCB.

Key words: umbilical cord blood, mononuclear cells, wheat grass extract, NK cells, flow cytometry

Introduction

Mononuclear cells (MNCs) of human umbilical cord blood (hUBC) are somewhat unlike adult peripheral MNCs. In peripheral blood, MNCs are vulnerable to immunological stressors. Most of the MNCs might have been immunologically primed as they were exposed to bio-stressors while MNCs from cord blood are not. Accordingly, hUBC MNCs are minimally primed, therefore, they are suitable for investigating the immunomodulating effects after treatment with natural health substances. During pregnancy, MNCs of hUBC are isolated from bio-stressors by virtue of the trophoblast stratum between the placenta and the neighboring maternal endometrium. This trophoblastic layer prevents MNCs of hUBC from being affected by various factors circulating in the maternal blood. We devised a model system (Figure 1) to use it in disclosing the lively and vigorous development of cellular differentiation of MNCs of hUCB after treatment with various extracts of natural substances. Immunal responses in the profile of immune cell subsets would be significant findings. The differentiation and maturation of MNC subtypes can be observed with flow cytometry using specific fluorescent monoclonal antibody staining[1].

The human immune system is composed largely of B cell-mediated response and T cell-mediated response. B cells make antibodies against soluble antibodies, while T cells are white blood cells and also known as lymphocytes. The NK cells are a special type of lymphocytes. Its natural cytotoxicity ability diminishes in the peripheral blood of patients who suffer from a variety of cancer, as contrasted to those of healthy controls[1]. Conversely, the cytolytic activity of purified umblilical cord blood (CB) NK cells was reported to be similar to that of purified adult peripheral blood NK cells[2]. It is known that the

Figure 1. Model of systemic approach from UCB cells to Stimulated cells

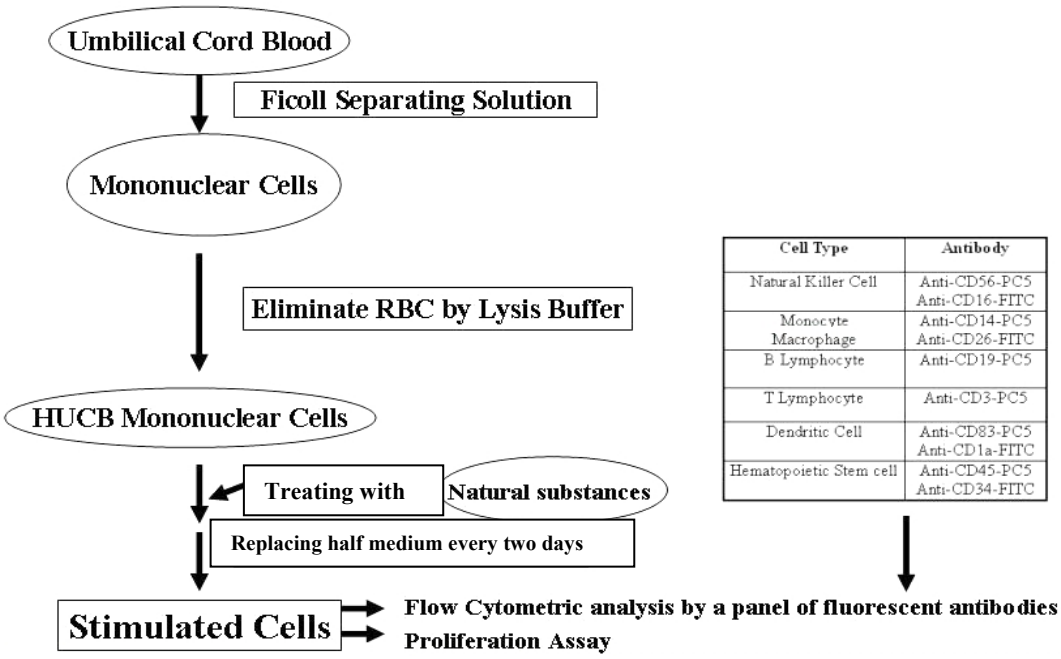


Figure 1, Model of systemic approach from UCB cells to stimulated cells. When an immune response is effected, various subsets of immune cells can be distinguished via their protein expression profiles. Through the use of specific fluorescently-labeled monoclonal antibodies and fluorescent-activated cell sorting, the differentiation and maturation of MNC subtypes can be characterized and quantified.

mature NK cells express CD56 alone or in combination with CD16 (CD is the abbreviation of the cluster of differentiation). CD antigens are expressed on cells of myeloid and lymphoid lineages. As most disease processes involve immune system activation or suppression, these antigens offer unique opportunities for monitoring host responses. The majority of adult peripheral blood NK cells are $CD56^{+}16^{+}$, with a minor population of $CD56^{+}16^{-}$ NK cells. In this study we also tested the effects of wheat grass extract on the NK cell surface marker expression in MNCs isolated from the UCB of six volunteers.

Natural Cytotoxicity

The decrease in levels of natural cytotoxicity in the peripheral blood of patients with various types of cancer is more evident than that in healthy adults[3]. Polysaccharides isolated from *Ganoderma lucidum*, a fucose-containing glycoprotein fraction (F3), enhances the cytotoxic activity of NK cells[4]. F3 effects the immunophenotypic expression in mononuclear cells (MNCs). When hUCB MNCs were treated with F3 (10-100 mg/mL) for 7 days, the population of $CD14^{+}CD26^{+}$ monocyte/macrophage, $CD83^{+}CD1a^{+}$dendritic cells and $CD16^{+}CD56^{+}$NK cells were 2.9, 2.3 and 1.5 times respectively higher than those of the untreated controls. The B-cell population had no significant change hitherto. The T cell growth was, however, slightly inhibited and the CD3 marker expression decreased about 20% in the presence of higher concentrations of F3 (100 μg/μml). F3 is not harmful to human cells *in vitro*, and after F3 treatment, NK cell-mediated cytotoxicity was significantly enhanced by 31.7% at effector/target cell ratio (Effector cells/Target cells, E/T) 20:1, but was not altered at E/T 5:1. In one of our other studies[5], a fucose-containing glycoprotein fraction (F3), isolated from the water-soluble extracts of *G. lucidum*, has shown the capacity in stimulating mice spleen cell proliferation and cytokine expression.

Flow Cytometry

Flow cytometry uses the principles of light scattering, light excitation and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5μm to 40μm diameter. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source (Figure 2). Lasers are most often used as a light source in flow cytometry.

The Eleven Natural Health Substances

The natural health substances include quercertein dehydrate, astaxanthin, CK-1 (ginsenoside compound-K), TCS-W (cordyceps sinensis), caffeic acid, bilobalide, eugenol, rutin hydrate, GSPE (grape seed proanthocyanidin extract) and wheat grass extract (Table 1). They

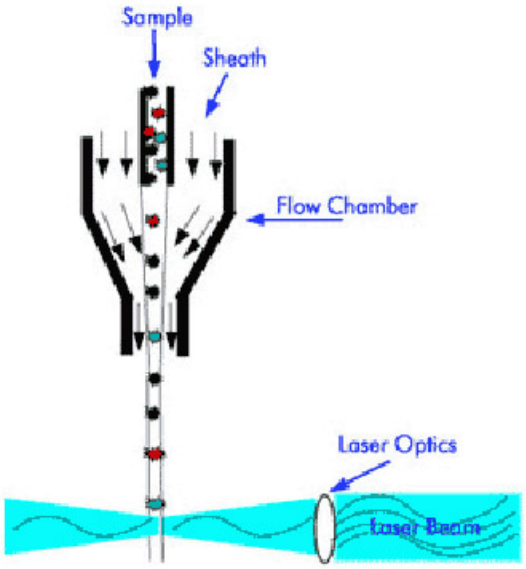
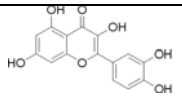
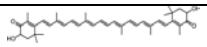
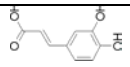
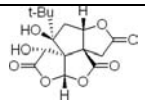
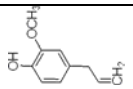
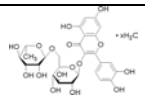


Figure 2, Model of systemic approach from UCB cells to stimulated cells. When an immune response is effected, various subsets of immune cells can be distinguished via their protein expression profiles. Through the use of specific fluorescently-labeled monoclonal antibodies and fluorescent-activated cell sorting, the differentiation and maturation of MNC subtypes can be characterized and quantified.

are considered to be antioxidants. Among them GSPE contains 5,000 ppm of resveratrol and is said to facilitate oxidant-induced vascular endothelial growth factor (VEGF) expression in keratinocytes as well as to be able to up-regulate hydrogen peroxide. *G. lucidum* has a fructotose-containing glycoprotein fraction (Table 1). Wheat grass extract is produced by sprouting and planting the seed of the common wheat plant, *Triticum aestivum*. Young leaves of wheat grass are crushed to create a juice or dried to make a powder. The unprocessed wheat grass contains high levels of cellulose, which makes it indigestible. Wheat grass possesses chlorophyll, dietary fiber, amino acids, minerals, vitamins, and enzymes. As the chlorophyll molecule is similar in structure to hemoglobin, leading some to believe that wheat grass helps blood flow, digestion and general detoxification of the human body. These claims have neither been proven nor disproven. Conversely, crested wheat grass, *Agropyron desertorum*, with a deep and fibrous root system is used for drought resistance. The use of wheat grass extract for treatment of various gastrointestinal disorders, especially for ulcerative colitis, along with some other conditions had been suggested by its proponents for more than 30 years. Wheat grass extract demonstrates a prominent tracing in cyclic voltammeter methodology, presumably corresponding to compounds that exhibit anti-oxidative properties. Under optimal reaction conditions, wheat grass peroxidase catalyzed the oxidation of certain aromatic amine substrate. Wheat grass, alfalfa and barley grass are green plants that many people also believe are holistically beneficial. Although, they are all a good source of chlorophyll that has some antibacterial effects in general, there is no evidence to support most of the claims made about these products.

Materials and Methods

This study was approved by the Internal Review Board of the Hospital where the babies were delivered. The study conformed to the Declaration of Helsinki. The authors report no conflict of interest. Each volunteer gave a written informed consent. Human UCB from six healthy volunteers were drawn into ethylenediamine tetraacetic acid (EDTA) coated tubes. The blood was collected after the full-term baby was delivered and before the placenta was separated from the uterus. Using aseptic procedures, an 18-gauge needle was inserted into the umbilical vein and umbilical cord blood was drawn for tests. The UCB (50 - 100mL) was processed using density gradient centrifugation with Ficoll-Paque (density 1.077; Pharmacia Biotech; Uppsala, Sweden). The buffy coat interface was retrieved and washed with Dulbecco's phosphate buffered saline (PBS, pH 7.4) and EDTA (0.2 mM). It was resuspended in a

Table 1 Summary of 11 Substances			
Compounds	**Molecular Weight**	**Structure Formula**	**Functions (both documented**
Quercetin dihydrate (qu)	338.27		Antioxidant, anti-inflammatory, anti-tumor, Anti-allergic, lower-cholesterol
Astaxanthin (as)	596.84		Antioxidant, anti-inflammatory
Caffeic acid (caff)	180.16		Antioxidant, anti-inflammatory, antihyperglycemic
Bilobalide (bilol)	326.30		Glutamate antagonism, neturoprotection
Eugenol (Eugen)	164.20		Antioxidant, anti-inflammatory
Rutin hydrate (Rutin)	610.52		Antioxidant, anti-inflammatory, anticarcinogenic, antithrombotic
Wheat grass extracts (wheat)			Antioxidant - wheat grass peroxidase catalyzed the oxidation of certain aromatic
Grape seed extract (GSPE)			Antioxidant containing 5000 ppm resveratrol facilitates oxidant-induced VEGF_expression in keratinocytes. GSPE
Ganoderma lucidum (a fructotose-containing glycoprotein fraction, F3) peroxide)			Antioxidant, an active glycoprotein component, isolated from the water soluble Reishi extract, designated fraction 3 (F3) is significantly active in stimulating mice spleen cell proliferation and cytokine expression.[4] The immunophenotypic effect of F3 on human immune cells has not yet been well documented.
CK- 1 (Ginsenoside Compound-K)			Antioxidant, The dicarboxylic acid ester derivatives of ginsenosides can be used to form pharmaceutical acceptable salts thereof having a high water solubility or can be directly dissolved in an aqueous solution of metal salt, and retain the pharmaceutical activities of ginsenosides said of such as tumor growth inhibition and cancer preventive cytotoxicity.
Cordyceps sinensis extract TCS-W			when used orally and appropriately. Cordyceps and a specific cordyceps preparation (CordyMax Cs-4) appear to be safe when used orally, short-term PREGNANCY AND LACTATION: Insufficient reliable information available; avoid using. Antioxidant

complete culture medium in RPMI-1640, consisting of L-glutamine and antibiotics (Gibco BRL), and was then supplemented with 20% fetal bovine serum (FBS). MNCs isolated through these procedures were prepared at a final concentration of 1 x 10^6 cells/mL. It is noted that RPM-1640 is a medium developed by Moore *et al.* at Rosewell Park Memorial Institute, USA.

Cells were cultured for 7 days after being treated with extracts of natural substances. Cells (1 - 2x10^6) were pelleted and resuspended in staining buffer (0.2 mM EDTA, 2% FBS in PBS). Staining buffer solution (100μL) containing 10μL of fluorescence-conjugated antibody was joined to the cell suspension for labeling. After incubation time at 4°C all samples were centrifuged and the pellets with rinsed buffer.

K562 (CCL-243, American Type Culture Collection), a human erythroleukemia cell line, was used as an NK-sensitive target for the cytotoxicity assays. These cells were cultured in RPMI-1640 medium (Gibco Laboratories) containing FBS and antibiotics (Gibco) in culture flasks (Falcon). On the day of testing, cells were rinsed with PBS and re-suspended.

NK-cells were treated with the wheat grass extract at one of the seven different concentrations ranging from 10 to 500 μg/mL for 7 days. Cytotoxicity analysis was done at the effector/target cells ratios of 5:1, 20:1, and 80:1.

Flow cytometry was performed with a FACSclibur cytometer (Becton Dickinson). The instrument was set for two-colour analysis using FACScomp software and was calibrated using Calibrite beads (Beckton Dickinson). Data were collected and analyses were performed using CellQuest software version 3.1f (Becton Dickinson).

The separation of $CD56^+$ NK-cells from other UCB MNCs was performed by a positive magnetic-bead cell separation method (MACS, Miltenyi Biotec). MNCs were segregated from the buffy coat of hUCB with Ficoll-Paque. Cells were then passed through 30 μm nylon mesh (Milipore). Filtered cells were rinsed with buffer PBS. The cell pellets were placed in 500 μL of the same buffer with the addition of 200 μL of FcR Blocking Reagent (Miltenyi Biotec) and incubated on ice to block Fc region (FcR). Two hundred μL of $CD56^+$ microbeads per 10^8 total cells were added and followed by supplementary incubation on ice. The cells were re-perched in the buffer solution. The magnetically bound cells were applied to two MACSy RS1 separation columns (Miltenyi Biotec). These columns had been equilibrated with the buffer in the magnetic field of the Vario MACSy separator according to the method of Gritzapis *et at*[6].

Aliquots of the cells were stained by PC5-labeled anti-$CD56^+$ monoclonal antibody (Coulter Immunotech, USA) to analyze the purity of $CD56^+$ NK cells which was at the level of 95%. All monoclonal antibodies to surface antigens, CD14, CD16, CD26, and CD56 (Fluorescein isothiocyanate, Serotec) were obtained from Coulter Immunotech, USA.

Cell numbers were observed using light microscopy, based on the ability of living cells to exclude trypan blue. Cell proliferation was evaluated by their reducing activity on sodium (2,3)-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, inner salt (XTT)[7,8]. One hundred μL of 2x10^5 cells/mL were incubated with different concentrations of the wheat extracts for 48 hours. Absorbance was measured with a spectrophotometer, using test and reference wavelengths of 450 and 650 nm, respectively.

The control group was treated with PBS. The $CD56^+$ NK-cell suspensions were cultured in a medium supplemented with RPMI-1640 medium with FBC for 24h (37^{o}C, 10% CO_2). Different concentrations of wheat grass extract, ranging from 10 to 500 μg/mL, prepared by serial dilutions, and those of positive control were respectively added to cell suspensions for preincubation treatment prior to the subsequent cytotoxicity assay. A positive control was prepared by using a prototype tumor promoter 12-O-tetradecanoyl phorbol-13 acetate (TPA), 10 and 1 μMol respectively. The results of wheat grass extract on $CD56^+$NK-cell cytotoxicity were expressed as ratios of survival of K562 cells of such natural-substance-treated groups versus the controls.

The MCNs isolated from the six hUCB specimens were placed in six T75 culture flasks at 5x10^5 cells/mL density in preparation for treatment. After seeding cells, the flasks were maintained in a 37°C, 5% CO_2 incubator for one hour to equilibrate before 100 μg/mL of natural substances were added to each culture. The extracts of natural substances including, but not limited to wheat grass were dissolved in PBS for all experiments.

Cell proliferation was evaluated by their reducing activity on WST-1 (4-3-4-Iodophenyl-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate). Basically, 100μl of 4x10^3 cells/well were incubated for 48 hours at 37^{o}C and 5% CO_2.

WST-1 reagent (Roche) was added at a level of 10µl/well and incubated for a further 4 hours at 37^{o} C and 5% CO_2. Absorbance was checked with a spectrophotometer, using test and reference wavelengths of 450 and 630 nm, respectively.

The number of lyses of target cells was revealed by the Alamar Blue assay. Alamar Blue indicator was added to the culture wells at a ratio of 200 µL of indicator to 2 mL of medium at twelve hours after the co-culture of effector and target cells. The cultured plates were incubated for 4 hours at 37°C. The absorbance of colors was measured on an ELISA reader at wavelengths of 570 nm and 595 nm controls, which contain only medium and Alamar Blue reagent that had been incubated for 4 hours were measured at the same wavelengths. It is noted that Alamar Blue, a colorimetric indicator, changes its colors from an oxidized (nonfluorescent, blue) to a reduced (fluorescent, red) form after being taken up by cells. In this process of experiment, Alamar blue was used for detecting target cell survival after treatments with effector cells and/or positive controls.

Cells were seeded by using pipettes and were cultured for 24 hours before adding the 11 nature substances to a final concentration of the natural substances of 100µg/ml. The duration that the cells were exposed to the substances was 7 days. The concentration of the solution before cells were harvested was 1,000,000/ml.

The ratio of sample % vs. control % of cell gated of 1.50 is considered remarkable while values of 2.0 are regarded as highly remarkable. Remarkable and highly remarkable are used in this article for easy referral by the readers. Their usage purposefully is to be distinguished from what has been usually and customarily referred to as statistically significant and highly significant.

The natural health substances used in this study include: quercetin dehydrate, astaxanthin, caffeic acid, bilobalide, eugenol, rutin hydrate, wheat grass extracts, GSPE, *G. lucidum,* and CK-1 (Table 1).

Results

Flow chart of CD immunocharacters

The flow cytometric analysis of different lymphocytes cell subtypes of uUCB among 55 specimens treated with extract of eleven different natural substances was done. When an immune response is effective, various subsets of immune cells can be distinguished via their protein expression profiles. Through the use of specific flourescently-labeled monoclonal antibodies and Fluorescent-activated cell sorting can characterize and quantify the differentiation and maturation of MNC subtypes (Figure 1).

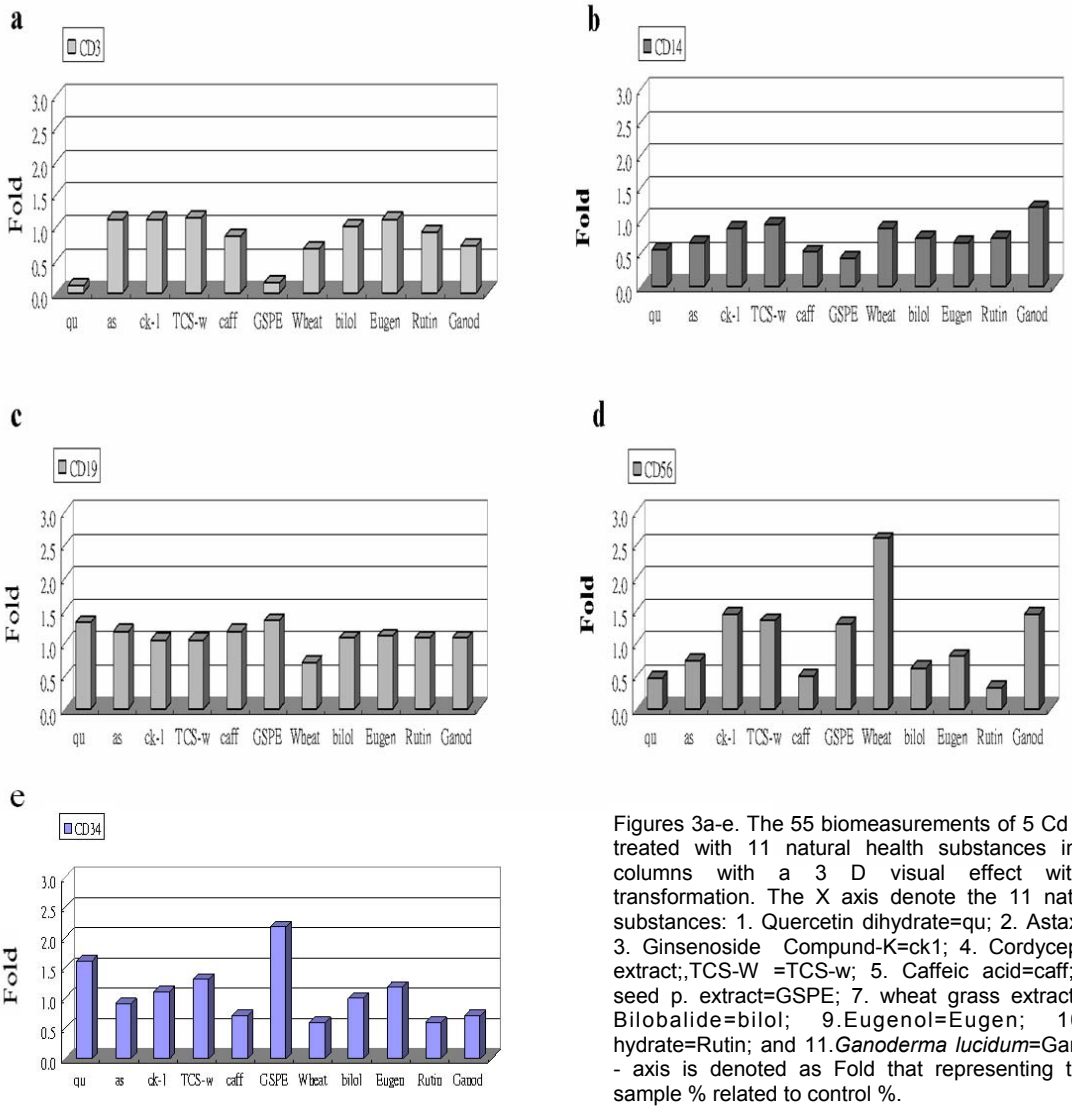
There were 55 measures for 11 nature substances. Each measure represents the ratio of sample % vs. control % . The mean ± SD of the total samples is 0.963 ± 0.4306. The 95% confidence Interval for Estimated Mean of Population was 0.845 to 0.117. The standard error of samples mean was 0.904 to 1.0211 in the ratios of survival of K562 cells of the treated groups versus the controls. For CD56+ NK cells, the ratio of samples % vs. controls % of wheat grass extract 2.23 was the highest (Fig. 3d,e).

Treatments with 11 different natural substances in cord blood mononuclear cells significantly altered the phenotypic expression of CD3+ T lymphocytes. Quercerin and GSPE significantly depressed the expression of CD3+ T lymphocytes/macrophage cell (Figure 3a). Treatments with different natural substances except *G. lucidum* (its ratio of sample % vs. control % is greater than 1.5) in cord mononuclear cells did not significantly alter the phenotypic expression of CD19+ B lymphocytes (Figure 3b). Treatments with different natural substance in cord blood mononuclear cells did not significantly alter the phenotypic expression of CD19+ B lymphocytes (Figure 3c). Treatments with different natural substances in cord blood mononuclear cells showed significantly altering the phenotypic expression of CD56+NK cell, with wheat enhancing the expression as the most effective (Figure 3d). Treatments with different natural substances in cord blood mononuclear cells showed significantly altering the phenotypic expression of CD34+ hematopoietic stem cells, with GSPE as the most effective (Figure 3e). Treatments with 11 different natural substances in cord blood mononuclear cells showed significantly altering the phenotypic expression of the 5 different CDs together at a glance in Figure 3f.

Among 55 measures there were 3 with relative high values: 2.3, 2.2 and 1.8. The Z score was obtained for the measure of 2.3 which was 3.80 times the standard deviation. The implication is that either this observation is actually outlier or the population distribution is far away from a normal distribution. Of importance in this context is the fact that 2.3 is the measure of ratio of wheat grass extract. This ratio of sample % vs. control % appears to out-perform as compared with that of the rest of ten natural substances.

There were 53 measures having values less than 2.0 while there were two measures having ratios greater than 2.0. Due to discrepancy of the number of these two different groups, Fisher's exact probability test was done. It reveals that the

Figure 3



Figures 3a-e. The 55 biomeasurements of 5 Cd monocytes treated with 11 natural health substances in clustered columns with a 3 D visual effect without data transformation. The X axis denote the 11 natural health substances: 1. Quercetin dihydrate=qu; 2. Astaxanthin=as; 3. Ginsenoside Compund-K=ck1; 4. Cordyceps sinensis extract;,TCS-W =TCS-w; 5. Caffeic acid=caff; 6. Grape seed p. extract=GSPE; 7. wheat grass extract=wheat; 8. Bilobalide=bilol; 9.Eugenol=Eugen; 10. Rutin hydrate=Rutin; and 11.*Ganoderma lucidum*=Ganod. The X - axis is denoted as Fold that representing the ratio of sample % related to control %.

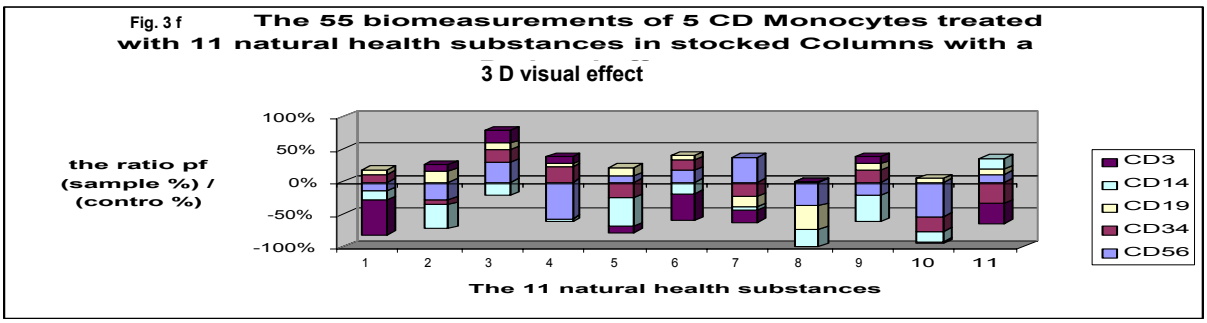


Figure 3f. The expression of lymphocyte markers revealed that for CD56 NK cells the ratio of samples % vs. controls % of wheat grass extract 2.30 is the highest. The flow cytometric analysis of MNCs in UCB revealed that the highest level of cytotoxicity was noted at an E/T ratio of 20:1 when the effector cells were pre-incubated with 10 μg/ml Wheat grass extract concentration. The experiments were done in triplicate. NK-cytotoxicity increased in 100 10μg/ml wheat grass extract by 29.09 times as compared with that of 10μg/ml ($p<0.01$).

difference between these two groups is statistically highly significant (two tailed p < 0.000003, degree of freedom = 53).

The results of the effect of wheat grass extract on $CD56^+$NK-cell cytotoxicity were expressed as ratios of survival of K562 cells of the treated groups versus the control. For CD56 NK cells, the ratio of samples % vs. controls % of wheat grass extract 2.3 was the highest exposure, which revealed a ratio of sample % vs. control % of cell gated, 1.51, and 1.613, respectively ($p<0.01$, Figure 4).

The results of wheat grass extract on $CD56^+$NK cell cytotoxicity were expressed as ratios of survival of K562 cells of the treated groups to that of controls (Figure 2). For $CD56^+$NK cells the ratio of sample % vs. controls % of wheat grass extract 2.30 is the highest.

Studies of the lysis of K562 cells after enrichment of $CD56^+$NK cells revealed that NK-cytotoxicity increased by 2.68 ($p< 0.05$) and 29.09 ($p<0.01$) after pre-treatment with 100 and 10 μg/mL of wheat grass extract, respectively, as compared to those of the untreated controls. The cytotoxicity at an E/T ratio of 5:1 was not significant, compared to the controls. When E/T ratios were as high as 80:1, no high cytotoxic effect was observed. Such a situation is likely due to over-saturation of cell numbers (data not shown).

The NK-cytotoxicity increased significantly by the ratio of 1.51, and 1.613 ($p<0.01$) respectively when pre-incubated with 1 and 10 μML TPA (Fig. 4). The latter was used as the positive control and NK cytotoxicity enhanced by wheat grass extract was comparable to that of this positive control.

Regarding individual variance of cell subtypes, it was found the ratio of sample % vs. control was 2.30 for the concentration of 10 μg/ml wheat grass extract, while that for the concentration of 100μg/ml wheat grass extract was 29.09 (Fig. 3f). These data imply that the phenotypical changes of NK cells might be the cause of enhancement of the NK- cells. Conversely, the ratios of CD14CD56 NK cells sample % vs. control % for wheat grass extract in the concentrations of 100 and 500 μg/ml were 1.79, and 1.89, respectively. Both are quite close to each other, which implies that the peak of the effect is around the concentration of 100 μg/ml (Fig. 5). The largest response ratio was noted at the lowest level of CD14CD56 NK cells. The population of CD14CD56 NK cells was about 12.65 times higher in the concentration of 10μg/ml than that of 100μg/ml (29.09: 30 = 12.65: 1). Therefore, in the presence of higher concentration (100μg/ml) the CD14CD56 marker expression decreased by 92.1%.

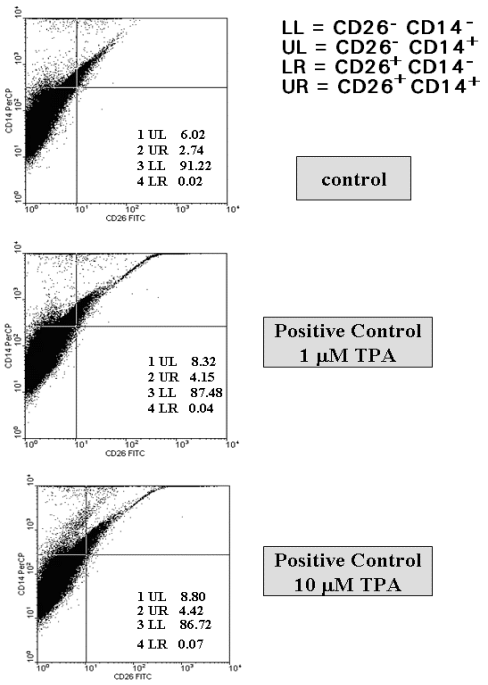


Figure 4. The different effects of $CD14^+CD26^+$ macrophages on the ratio of sample% vs. control% , with positive controls using TPA in different concentrations. Treatments were done under the exposure to a prototype tumor promoter 12-O-tetradecanoyl phorbol-13 acetate (TPA), 1 and 10 μM respectively were used as positive control, from which revealed a ratio of sample % vs. control % of cell gated, 1.51, and 1.613, respectively ($p>0.01$).

Regarding individual variances of cell sizes the ratio of sample% vs. Control % were greater than 1.5 for those with NK cells < 10 kDa fraction. The ratios were less than 1.5, respectively for CD16 CD56 + NK cells > 30 kda fraction and CD34 CD45 + stem cells/leukocytes for >30 kDa fraction, respectively (Figure 6).

Discussion

The statistical distribution of various CDs with the 55 biomeasurements before transformation with logarithm was determined (Table 2). Without transformation, the difference of distribution among the various CDs lies in 0the fact that these five CDs were in normal distribution. Upon Kolmongrov - Smirnov test, the entire 5 CDs were still all in normal distributions (Table 7).

The entire 55 measures after transformation with logarithm, for variables of all CDs, reveals that Normal distribution parameters estimated: mean = -0.15971796, and Standard Deviation = 0.53148599. The Kalmongrove-Smirnoff test was carried out in order to test normality: p value = 0.288, which is greater than the significant level of 0.05, therefore, the null hypothesis of

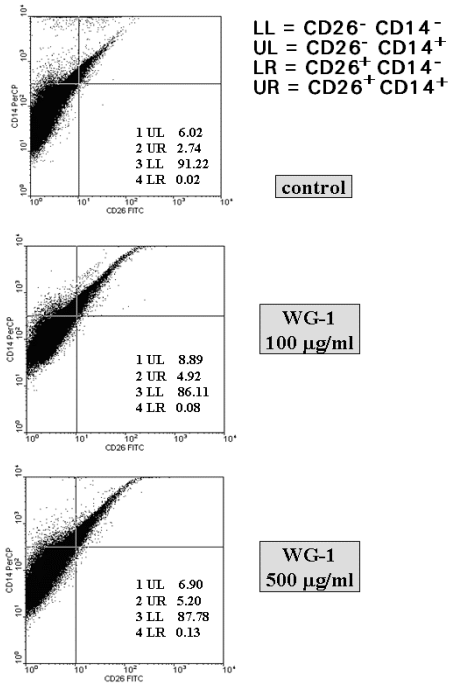


Figure 5. The differences in the ratios of sample % vs. control % of CD26CD14 monocytres/macrophages when using different wheat grass extract concentrations of 100, and 500 µg/ml, respectively.

Kalmongrove-Smirnov test is not rejected. The distribution is normal (Figure 7).

The out-performance of CD56 NK cells treated with wheat grass extract

The explanation of more than two SD away from the mean is as follows: the measurement of 2.3, as the effect of treatment with wheat grass extract on CD56, after logarithmic transformation is not a statistical outlier of this distribution, but actually is the best performance among these 55 biomeasurements to the extent that the ratio of sample % vs. control % is concerned. Such an inference is further supported with Box-plots and Histogram after transformation with logarithm (Figure 8). Whereas two other measurements of the ratio of sample % vs. control %, for CD3 T lymphocytes, 0.11 treated with Quercetin, and that for the identical CD3 T lymphocytes, 0.16 treated with GSPE respectively are the actual outliers of this population distribution (Figure 8a Box-plot, the most right handed plot, the two datum points below this red box, and in Fig 8b, the two isolated columns situated at the most left handed site in the blue Histogram.) Of importance in this context is the fact that 2.3 is the measure of ratio of wheat grass extract. The ratio of 2.3 of sample % vs. control % does best out-perform as compared with the rest of 10 other natural substances, and not to be regarded as an outlier.

Lyses of K562 cells after enrichment of $CD56^+$ NK cells vs. that after the treatment with the positive control TPA

NK-cytotoxicity increased significantly by the ratio of 1.51, and 1.613 ($p < 0.01$) respectively in pre-incubation with 1 uML and 10 uML TPA, which is the positive control (Figure 4).

Individual variance of cell subtypes

For wheat grass extract, in the concentration of 100ug/ml, the ratio of sample % vs. control was 2.68, while that in the concentration of 10ug/ml was 29.09. These data may indicate that the phenotypical changes of NK cells might be the cause of enhancement of the NK-cell cytotoxicity effect, and treatment with high concentrations of wheat grass extract may decrease the NK cell growth (Figure 5). Our impression is as following: for samples treated with wheat grass extract, on $CD56^+$ NK cells, the ratio of sample % vs. control % was 2.3, the highest among all the 55 measures. We hence believe that wheat grass extract was advancing cellular immunophenotypic expression, and promoting the cytotoxicity of $CD56^+$ NK cells.

FcR Receptor blocking in Fc regions (FcR)

During the experimental process of using magnetic labeling, microbeads were conjugated to monoclonal antibodies that have exposed Fc regions (FcR). At such regions there was binding taking place non-specifically to FcR receptors. Hence, certain FcR blocking reagent is used to block unwanted binding of antibodies to human cells expressing Fc receptors such as B cells, monocytes, and macrophages. With these receptors blocked, the microbeads will only bind to their target antigen, thus increasing the specificity of the magnetic labeling.

The Fc receptor block reagent does not alter the binding behaviour - like affinity, avdity etc. - of the antibodies (e.g. microbeads or fluorochrome conjugate). The expectation of increasing of specificity means in this case that unwanted binding to Fc receptors is subdued. Under our observation, in many cases e.g. rare cell isolation adding or leaving out certain Fc receptor reagent, showed different results in the flow cytometry examination. Without Fc receptor blocking reagent, negative cells

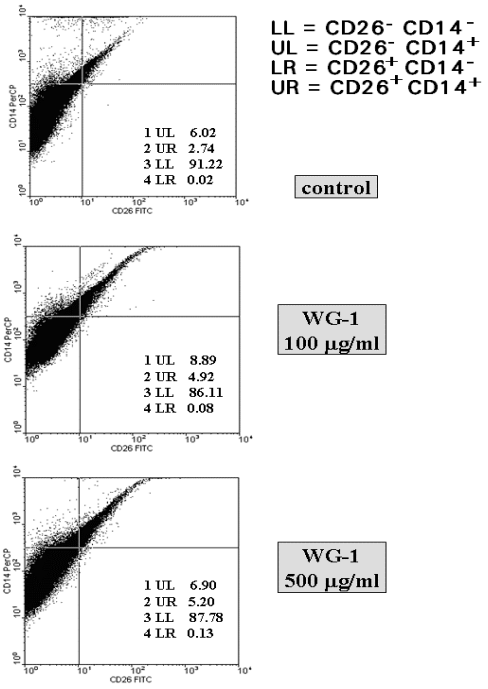


Figure 5. The differences in the ratios of sample % vs. control % of CD26CD14 monocytres/macrophages when using different wheat grass extract concentrations of 100, and 500 µg/ml, respectively.

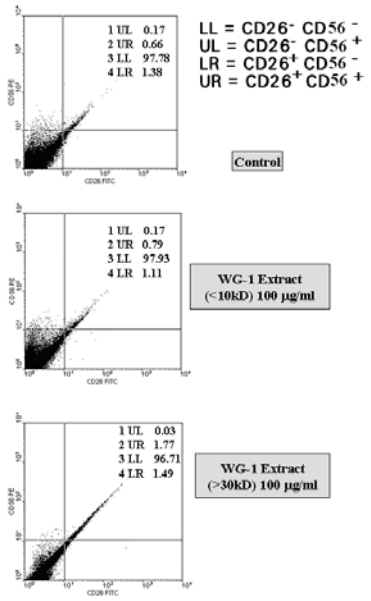


Figure 6. The differences on $CD16^{+}CD56^{+}$ NK cells in ratios of sample% vs. control %, segmented by different fractions of cell size, >30 kDa vs. < 10 kDa, respectively.

Table 2. The p values of various CDs after Kolmogorov-Smirnov test,

CD types	CD56	CD34	CD19	CD14	CD3
MNC types	NK cells	Hematopoietic stem cells	B lymphocyte	Moncytes/Macrophages	T lymphocyte
Testing for NORMAL DISTRIBUTION with Kolmongrov - Smirnoff test	0.588	0.939	0.307	0.891	0.824

The p value was set at the level of 0.05; if less than 0.05, then denoting significant. Five CDs, namely CD 56, CD 34, CD19, CD14, and CD3 are all with p value > 0.05 respectively, which is denoting that these 5 CDs are insignificant. Hence, they are in normal distribution upon Kolmongrov - Smirnoff test for determining normality.

can even shift when staining with a fluorochrome conjugate has been performed. Adding the Fc receptor blocking reagent did reduce or abolish such a shifting.

Fc receptor blocking is, at least in immunology, a widely accepted standard method, especially when we are working with rare cells, such as CD56 NK cells. Blocking reagents against Fc receptors is a standard routine for all staining performed. It is done to be absolutely certain that the positive staining signal is really due to the specific binding of the antibody to its recognized antigen and not just the Fc part binding to the Fc receptor. In this study the cell source was a pregnant woman immediately after delivering baby, which may contain less Fc receptor positive cells or cells with less up-regulated Fc receptors than from a patient in general.

The limitations of the Study

The lack of using purified compounds but instead employing the crude extracts in the natural substance assay is inherently a limitation of this study. It is noted that NK-T cells comprise less than 0.1% of

Figure 7: The Quantile - Quantile Plot of the 55 Biomeasurements

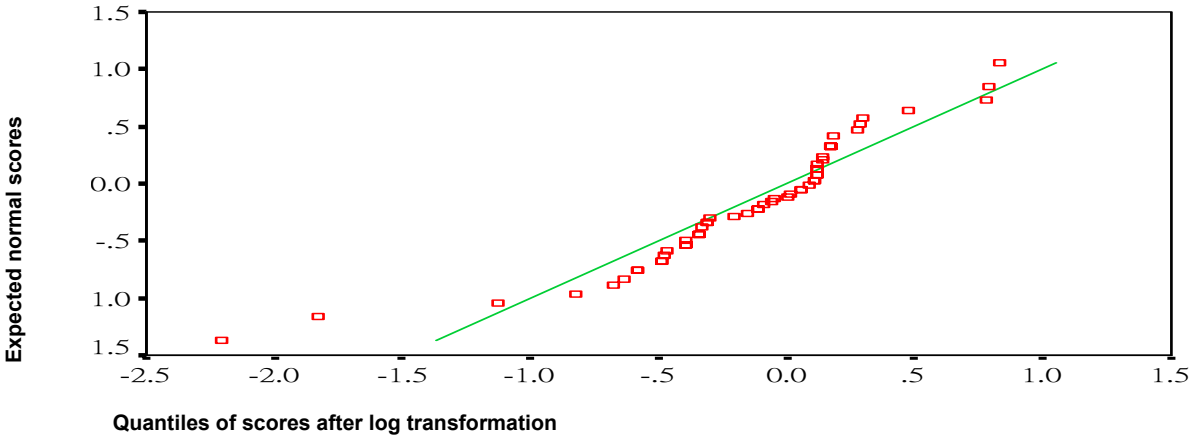


Figure 7. Scatter plot with the quantiles of scores after logarithmic transformation of the horizontal axis and the expected normal scores on the vertical axis. The steps in constructing a QQ plot are as follows: first, the data are sorted from smallest to largest. A plot of these scores against the expected normal scores should reveal a straight line. The expected normal scores are calculated by taking the z-scores of (I -½)/n where I is the rank in increasing order. Before transformation with logarithm, a Kolmogrove-Smirnov test was done. The p-values of various CDs were tabulated in Table 2. For variables, all CDs of entire 55 measures (after taking log-transformation), the parameters are as following: Normal distribution parameters estimated: mean = -0.15971796, and Standard Deviation (SD)= 0.53148599. After performing the Kalmongrove-Smirnov test to ascertain normality: p value = 0.288, which was greater than 0.05, the significant level set. Curvature of the points indicates departures of normality. This plot is also useful for detecting outliers. The outliers appear as points that are far away from the overall pattern of most points involved[9].

Figure 8a,b: BOX-PLOT and Histogram status post logarithmic transformation

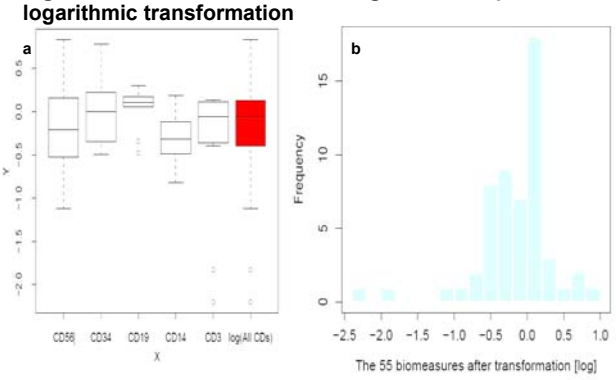


Figure 8a. X-axis: five different CDs treated with 11 natural health substances from left to right in the order of A) CD56, B) CD34, C) CD19, D) CD14, and E) CD3, while the most right sided column representing. Y-axis: for the measurement of the ratio of sample % vs. control % treated with 11 natural substances. Of importance in this context is the fact that 2.3 is the measure of ratio of wheat grass extract. Such a ratio of 2.3 of sample % vs. control % does best out-perform as compared with the rest of 10 other natural substances in this pilot study, and not to be regarded as any outlier. Among totally 55 such measures, 2.3 is the value of CD56 NK cells treated with wheat grass extract, as being indicated. Whereas two other measurements of the ratio of sample % vs. control %, for CD3 T lymphocytes, 0.11 treated with Quercetin, and that for the identical CD3 T lymphocytes, 0.16 treated with GSPE respectively are the actual outliers of this population distribution, they are represented by the two isolated columns situated at the most left handed side in the Histogram. Hence, the UCB samples from six different individuals (participants) after treatments with wheat grass extract respectively exhibited a significant immune response, predominantly involving CD56 NK cells in the current study. b. X-axis: five different CDs from left to right in the order of A) CD56, B) CD34, C) CD19, D) CD14, and E) CD3, while the most right sided box is representing the entire 55 biomeasurements. Y-axis: for the measurement of the ratio of sample % vs. control % treated with 11 natural substances. Among totally 55 such measures, 2.3 is the value of CD56 wheat grass extract, as being indicated value (2.3) of the most left box. Such a value does best performance as compared with that of the rest 10 other natural substances in this study. It is noted that the two datum points below this box belong to the measurements of CD3 T lymphocytes, as the outliers, so far as ge two point that are below the adjacent red box.

lymphocytes in adult peripheral blood and umbilical cord blood[10]. Conversely, both NK-cells and NK-T cells may play important roles in protecting the newborn against infection, which certainly deserves further study. In addition, a category of NK cells which are activated by post-interleukin 2 activity has distinctively been called Lymphokine-activated killer (LAK) cells. They have been reported to produce a soluble, yet unidentified proteins, in mixed tumor-adherent MNC cultures of PB, which substantially reduced the induction of LAK cells in the culture[11]. There are other proposals such as the existence of a postoperative immunosuppression cascade consisting of 1) increases in cytokines and 2) decreases of immunosuppressive proteins in both the helper and the cytotoxic T-cell populations. There is another suggestion for the development of suppressor T-cell activities postoperatively[12]. We have not yet addressed to these issues in the current report.

This study has 11 treatments with 1 sample of CD56 cells per treatment with one of each of the 11 natural health substances (one single number as an outcome per treatment), and same for each of the rest of 4 CDs. Unfortunately, because of a shortage of umbilical cord blood we do not have multiple measures within treatment variances, but merely one outcome per treatment. Hence, we could not do statistics on the differences in effects between treatments, but to merely express the finding in a purely descriptive manner. Noticeably, interleukin 12 can stimulate the activated Tc cells, and advances the differentiation of the NK cells[13]. Modulating

immunological functions in lymphocytes of umbilical cord blood with natural health substances is very important as well as interesting[14] and, if successfully accomplished, could advance our knowledge in controlling human defense against wide varieties of diseases. Nevertheless, the results presented in this report thus far still fall short of this aim.

Conclusion

In conclusion, the identification and purification of molecules of the crude wheat grass extract, which is likely active for augmenting CD56 NK cell function, may open a new avenue of cancer immunotherapy that stimulates innate immunity.

Acknowledgments

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The Leydig Organ: A Unique Lymphomyeloid Organ Of Elasmobranchs

by

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ABSTRACT

The anatomy and histology of the Leydig organ was studied in the smooth dogfish, *Mustelus cania* and the clearnose skate, *Raja eglanteria*. Histological sections were prepared for light microscopy from each species. The Leydig organ was found to be packed with myelo- and lymphopoietic tissue in both species. Reticular tissue was seen to permeate the stroma of the organ. In addition, sinusoidal spaces were apparent in the Leydig organ of the shark and skate. In *M. cania*, the organ was fully compartmentalized into sinusoids filled with blood-forming tissue. Less compartmentalization was seen in the Leydig organ of *R. eglanteria*. The phylogenetic significance of this organ is discussed.

Introduction

In higher vertebrates the primary hemopoietic tissues are located in the bone marrow[1,2]. Bone marrow contains erythropoietic, myelopoietic and lymphopoietic tissues[2]. Secondary centers for lymphopoiesis are found in lymph nodes, thymus and spleen[1]. Lacking bone marrow and lymph nodes, elasmobranchs rely on two structures unique to their class, the Leydig and epigonal organs for lymphomyelopoiesis[3 - 7]. Depending on the species of elasmobranch, the epigonal organ is found either within[1] or adjacent[6] to the gonads. This organ closely resembles the Leydig organ and is unique to cartilaginous fishes[3,8]. Most elasmobranchs possess both structures while some have only one[4]. Based on structure and types of cells within these tissues it is agreed that the Leydig and epigonal organs function as bone marrow (although erythropoiesis is not observed) and lymph nodes of higher vertebrates[8,9].

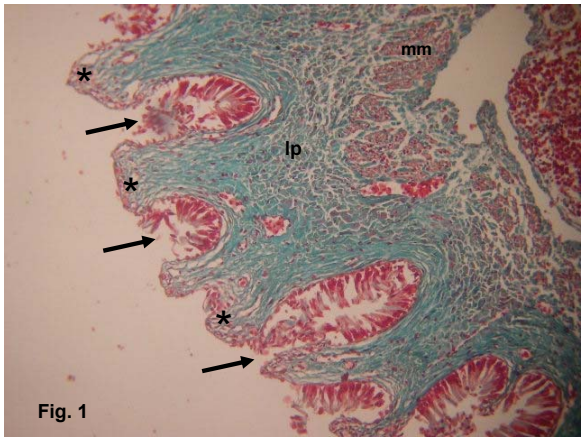
The first description of the Leydig organ in the literature was made by Steno in 1685 (as cited by Mattisson and Fänge[2]). He described this organ as a gland-like structure in the wall of the esophagus of the ray. In 1857, Leydig [10] described this mass of tissue as a lymphatic organ. Subsequently, it was referred to as the Leydig organ[2]. Fänge[11] has found well developed Leydig organs in every species of elasmobranch he studied. From a number of investigations, it has been determined that the Leydig organ is composed of a ventral and dorsal segment which extends from the last branchial arch to the cardia of the stomach[1,6,8,11,12]. In this study, we describe the Leydig organ of two species of elasmobranch, *Raja eglanteria* (clearnose skate) and *Mustelus cania* (smooth dogfish) caught within the New York Bight during the summers of 2006 and 2007.

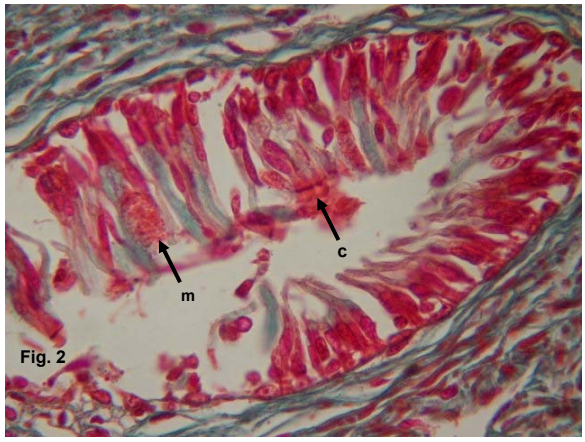
Anatomy

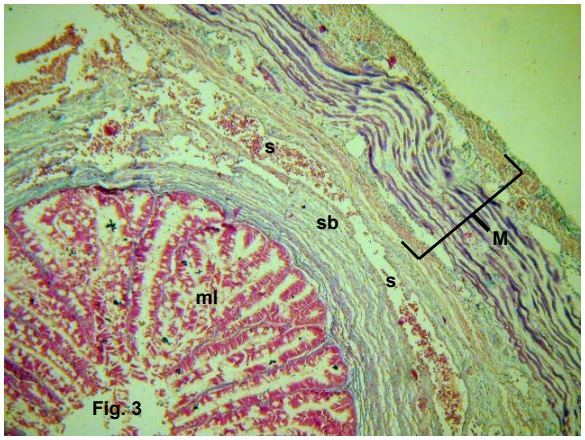
In *R. eglanteria* and *M. cania*, the Leydig organ can be found in the submucosa of the esophageal wall. It appears to be a more substantial organ in *R. eglanteria*. This difference could be species specific or related to the differences in age or health of the specimens. Since both fishes were caught at the same time of the year, seasonal differences can be eliminated as a reason for the larger organ in *R. eglanteria*. In both species, the stroma of the organ is primarily composed of granulocytic and lymphocytic cells.

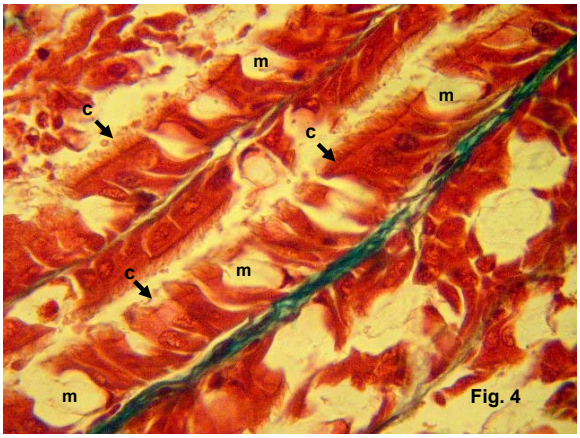
In *R. eglanteria,* the esophageal mucosal surface exhibits papilla-like projections alternating with rounded pits invaginating into the lamina propria. The pits are lined with a simple columnar epithelium (Figs.1 and 2). Mucus producing and ciliated cells are found in this epithelium (Fig. 2). The raised surfaces are covered with squamous epithelium which appears to be simple or, at most, a few cells in thickness (Fig. 1). In *M. cania*, the mucosal surface is deeply folded and completely covered with simple columnar epithelium (Fig. 3). The cell types in this epithelium are predominantly ciliated with interspersed mucus producing cells (Fig. 4). In both species, the lamina propria is richly endowed with connective tissue fibers. Small blood vessels and capillaries are commonly seen.

In both species, the Leydig organ is located under the mucosa and occupies most of the submucosa (Figs. 5 and 6). Sinusoidal spaces are found at this juncture. In *R. eglanteria*, these sinusoids are enveloped with connective tissue fibers and can be seen to extend into the stroma of the Leydig organ. Bands of fibrous connective tissue are woven throughout the Leydig organ stroma (Figs. 7 and 8). Occasionally, small blood vessels can be seen.









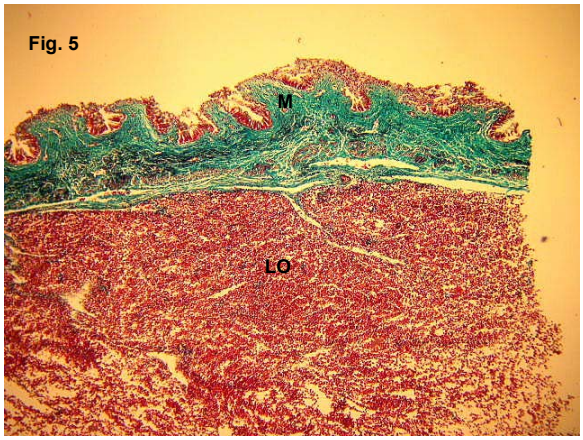
Sections through the esophagus containing the Leydig organ of the clearnose skate, *Raja eglanteria* and the smooth dogfish, *Mustelus cania* stained with Masson's trichrome.
Figure 1. Mucosal surface in *Raja eglanteria* showing pits (arrows) lined with simple columnar epithelium. Adjoining surfaces are lined with squamous tissue (*); lamina propria (lp), muscularis mucosa (mm).
Figure 2. *R. eglanteria*: Mucosal pit showing mucous cell (m) and ciliated cells (c).
Figure 3. *Mustelus cania*: Mucosal lining (ml), submucosa (sb), sinusoids (s), muscular layers (M).
Figure 4. Mucosal surface of *M. cania*: Ciliated cells (c), mucus producing cells (m).

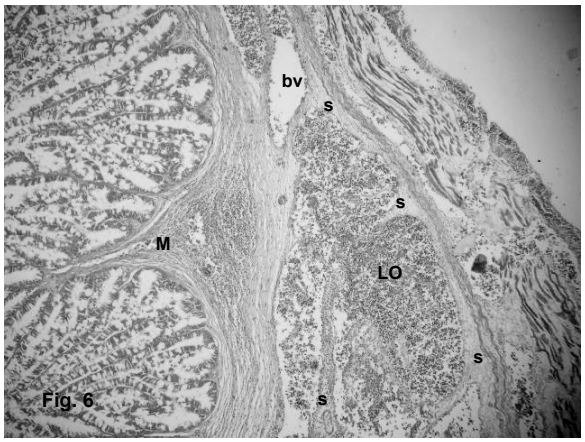
The Leydig organ of *M. cania* is more lobulated than that of *R eglanteria*. Thick connective tissue septae extend throughout the parenchyma, serving to subdivide the Leydig organ into a number of sinusoidal spaces filled with leucocytic tissue (Fig. 6). An occasional small blood vessel or sinusoidal space can be seen within the septae. Sinusoidal spaces also contribute to the lobulation of the gland (Fig. 9).

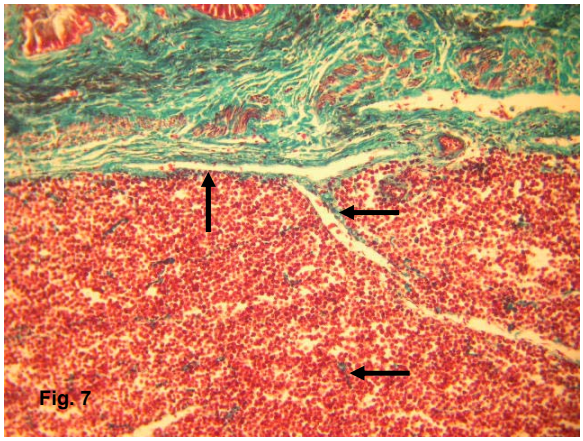
Histology

The cellular makeup of the Leydig organ in *M. cania* and *R. eglanteria* is essentially identical. The organ is densely packed with granulocytic (Fig. 10) and lymphocytic cells (Fig. 11). The granulocytes appear to be in different stages of maturation (Fig. 10). The presence of granulocytic and lymphocytic cell types in the Leydig organ has been noted in other studies[7,8]. No evidence of erythropoietic activity has been observed in our study of the Leydig organ and none has been observed in other investigations[3,8]. The primary hemopoietic activity of the Leydig organ has been reported to be the production of eosinophilic granulocytes[11]. The granules of these cells vary from coarse, round or oval inclusions to very minute rod-shaped granules[11].

Mattisson and Fänge[4] described two distinct populations of granulocytes within the Leydig organ, eosinophilic granulocytes and heterophilic granulocytes. According to Mattisson and Fänge[4], the granules of the heterophilic cells were smaller (0.5 μm) than the eosinophilc granules (1.5 μm) and tended to be rod-shaped. In this study, we were unable to differentiate the granulocytic cells into subtypes from our tissue sections. Future studies of the cell populations of the Leydig organ







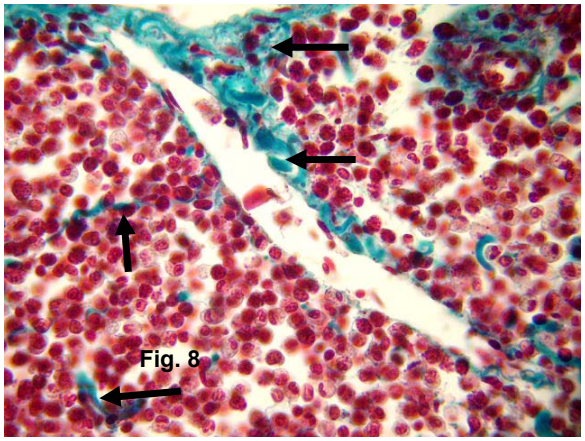


Figure 5. *R. eglanteria*: Mucosa (M), Leydig organ (LO).
Figure 6. *M. cania*: Mucosa (M), Leydig organ (LO), septa (s), blood vessels (bv).
Figure 7. *R. eglanteria*: Fibrous connective tissue (arrow) in Leydig organ.
Figure 8. *R. eglanteria*: A higher magnification of Fig. 7.

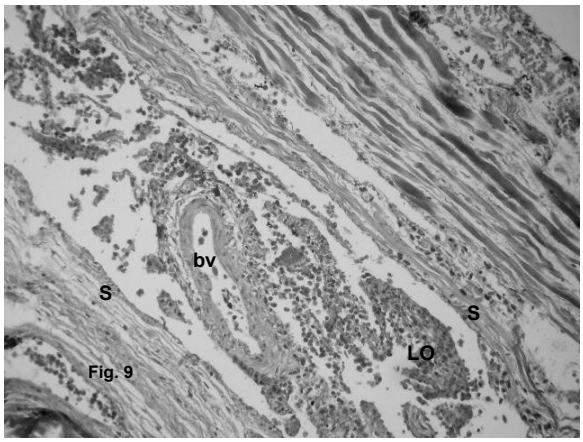
will be carried out using stained imprints of the tissue.

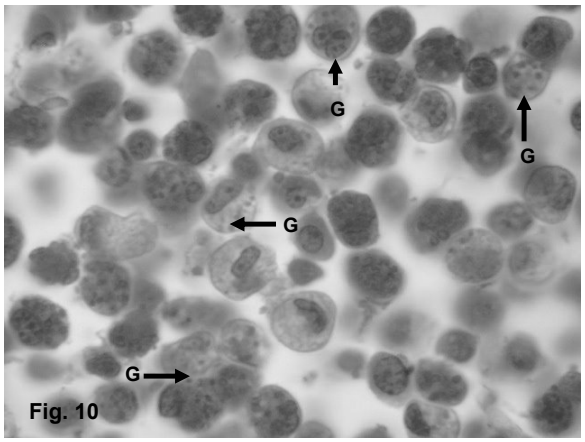
Beyond the Leydig organ, a very delicate layer of smooth muscle fibers can be seen (Fig. 12). Peripheral to this smooth muscle band lies a thick layer of circular skeletal muscle. A thin outer layer of longitudinal muscle is also present (Fig. 13). The entire circumference of the esophagus is surrounded by a serosa containing small blood vessels. Transected nerve fibers, presumably associated with the enteric plexuses, are often seen between the muscle fibers and the overlying serosa (Fig. 14).

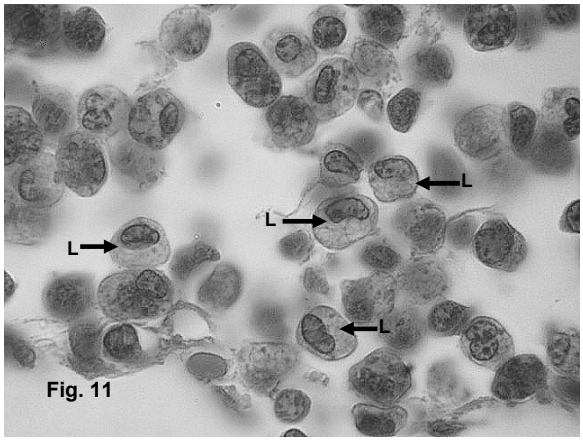
Discussion

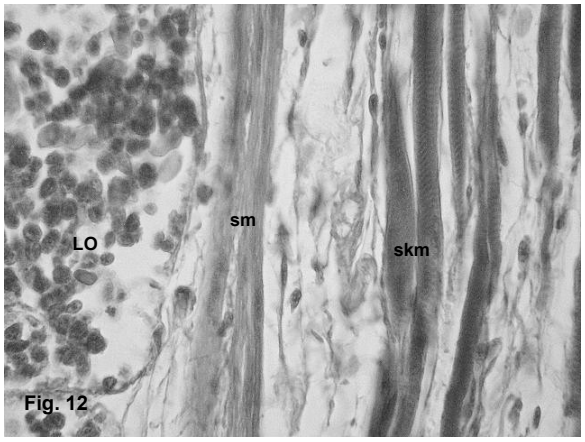
The significance of the location of the Leydig organ in the esophageal submucosa of elasmobranchs could be attributed to the fact that the digestive tract is a major portal of entry for microbes. The Leydig organ is strategically located to provide an immunogenic response to potential pathogens. In higher vertebrates, lymphoid nodules are generously distributed throughout the gut (GALT)[1,2]. Developmentally, in the dogfish, *Scyliorhinus canicula*, the appearance of the Leydig and epigonal organs coincides with the first exposure to seaborne microbes and other antigenic material. This occurs some six months after egg release when the egg case splits open[6].

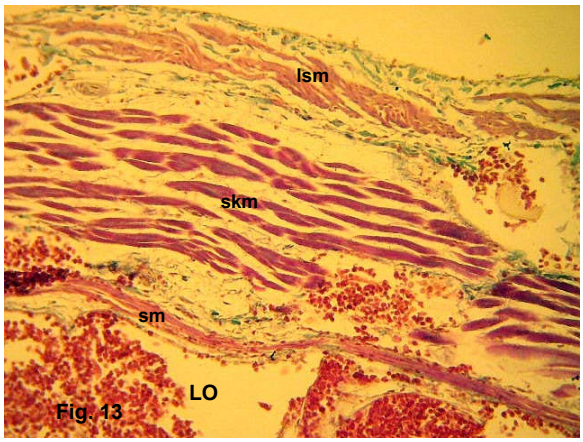
The Leydig organ has been described as being chiefly a myelopoietic structure. Yet, our investigation indicates a significant population of lymphocytic cells exists in this organ. It is possible that the lymphoid-like cells represent myeloblastic stages of granulocyte formation. Walsh and Luer[7] described the Leydig organ as being packed with granulocytic cells in various stages of maturation. Alternatively, it has been suggested that the origin of the lymphocytic cells of the Leydig organ may be the thymus gland[13]. In most elasmobranchs, the primary organ of lymphopoiesis is the thymus[6]. Since the thymus precedes the Leydig organ developmentally, it is reasonable to suggest that the source of Leydig lymphocytes could be the thymus gland.











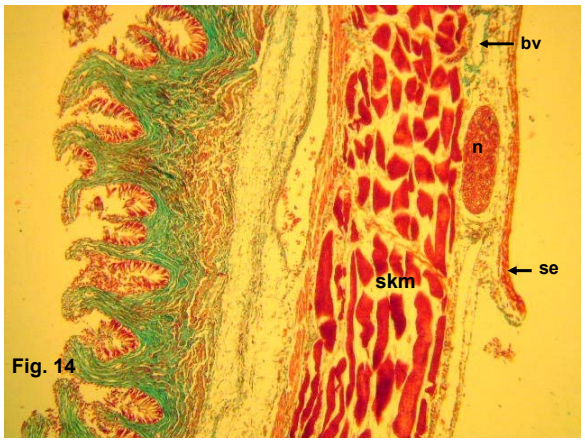


Figure 9. *M. cania*: Leydig organ (LO), septa (S), blood vessel (bv).
Figure 10. *R. eglanteria*: Parenchyma of the Leydig organ with granulocytic cells (G).
Figure 11. *R. eglanteria*: Parenchyma of Leydig organ with lymophocytic cells (L).
Figure 12. *M. cania*: Leydig organ (LO), smooth muscle (sm), skeletal muscle (skm).
Figure 13. M. cania: Leydig organ (LO), smooth muscle (sm), skeletal muscle (skm), longitudinal smooth muscle (lsm).
Figure 14. *R. eglanteria*: Skeletal muscle (skm), nerve (n), serosa (se), blood vessel (bv).

Lacking the centralized hemopoietic structures of higher vertebrates, bone marrow and lymph nodes, elasmobranchs carry out erythropoiesis, thrombopoiesis and myelogranulopoiesis in different locations[14]. Granulopoiesis and lymphopoiesis occur within the Leydig and epigonal organs of these forms[1]. In a few species such as *Scyliorhinus canicula*[6,15] and *Raja clavata*[3], the spleen functions in erythropoiesis and, to some extent, lymphopoiesis. Most investigators, however, suggest that erythropoiesis and thrombopoiesis occur within the general circulation of cartilaginous fishes[1,5].

The Leydig and epigonal organs could be a more primitive representation of hemopoietic and immune function from that found in higher phylogenetic forms. In the higher teleosts, granulopoiesis, erythropoiesis and thrombopoiesis take place in the kidney and, to some degree, within the spleen while lymphopoiesis takes place within the spleen and GALT (gut associated lymphoid tissue)[1]. In urodele amphibians, granulocytic loci occur in the liver and lymphopoietic loci are found, as in teleosts, within the spleen and GALT. Erythropoiesis occurs in the spleen of the urodele[1]. In the anurans, bone marrow becomes differentiated and serves as the site if granulopoiesis, erythropoiesis and thrombopoiesis while lymphopoiesis occurs in the spleen[1]. In the more advanced vertebrate classes the bone marrow has become differentiated so that both erythroid and myeloid functions have become confined to this area[1]. It appears that, phylogenetically, the sites of erythropoiesis and granulopoiesis, originally found in different loci in sharks, rays and skates have merged into a single hemopoietic site, the bone marrow of higher forms. Furthermore, the Leydig and epigonal organs of cartilaginous fishes provide a system in which to isolate the microenvironmental factors that separately affect the process of granulopoiesis and erythropoiesis.

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Effects of p-Aminosalicylic acid on the Neurotoxicity of Manganese and Levels of Dopamine and Serotonin in the Nervous System and Innervated Organs of *Crassostrea virginica*

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Abstract

Manganese is a neurotoxin causing Manganism in individuals chronically exposed to elevated levels in their environment. Toxic manganese exposure causes mental and emotional disturbances, and a movement disorder similar to Idiopathic Parkinsons Disease. Manganese interferes with dopamine neurons involved in control of body movements. Recently, p-aminosalicylic acid (PAS) is being used to alleviate symptoms of Manganism, but its mechanism of action is unknown. The eastern oyster, *Crassostrea virginica*, possesses a dopaminergic innervation of its gill. Oysters exposed to manganese have reduced levels of dopamine in the cerebral ganglia, visceral ganglia and gill, but not of norepinephrine, octopamine or serotonin. Those results are consistent with reported mechanisms of action of manganese in human and mammalian systems. In this study we determined the effects of PAS treatments on dopamine and serotonin levels in oysters exposed to manganese. Adult *C. virginica* were exposed to 500 μM and 1 mM of manganese with and without 500 μM and 1 mM of PAS by removing one shell and maintaining the animals in individual containers of aerated artificial sea water at 18º C for 3 days. Control animals were similarly treated without manganese or PAS. Dopamine and serotonin levels were measured by HPLC with fluorescence detection. PAS protected the ganglia and gill against the effects of 500 μM manganese, but not against the 1 mM manganese treatments. Serotonin levels were not affected by the treatments. The study demonstrates PAS can protect against reductions in dopamine levels caused by neurotoxic manganese exposure, but is concentration dependent. These findings may provide insights into the actions of PAS in therapeutic treatments of Manganism.

Introduction

Manganese is present in animal tissues and is required as an enzyme cofactor or activator for numerous reactions of metabolism[1]. While essential in trace amounts, excessive manganese exposure can result in toxic accumulations in human brain causing extrapyramidal symptoms similar to those seen in patients with Idiopathic Parkinson's disease[2-5], a dopaminergic cell disorder. This Parkinson-like neurological condition was first described in 1837 in two manganese ore-crushing mill workers[6] and has since been referred to as Manganism[7-10]. Symptoms common to both disorders include gait imbalance, rigidity, tremors and bradykinesia[7,11-13], suggesting a similar etiology of neuronal damage in the substantia nigra with a resulting deficiency of the neurotransmitter dopamine for the striatum. However, compared to Parkinsons disease, there are some differentiating features seen with Manganism including symmetry of effects, more prominent dystonia, a characteristic "cock walk," an intention rather than resting tremor, earlier behavioral and cognitive dysfunction, difficulty turning, and a poor response to Levodopa[2,14-20], suggesting different or more extensive damage in the basal ganglia or to the dopaminergic system.

The primary cause of manganese toxicity is believed to be by inhalation of manganese from the atmosphere[21]. In addition to mining and manganese ore processing, high levels of airborne manganese are possible in a number of other occupational settings, including welding, dry battery manufacture and use of certain organochemical fungicides like Maneb[14, 22-25].

Although manganese toxicity has been recognized for some time, the primary mechanism underlying its neurotoxic effects remains elusive. Human and animal studies have shown that toxic exposure to manganese results in metal accumulations in various areas of the basal ganglia and dysfunction of cells of both the striatum and the globus pallidus[2,26-32]. Other studies have shown that manganese selectively targets dopaminergic neurons in the human basal ganglia[14,31] and decreases dopamine levels in the striatum[11,27,33-35]. Considering the clinical similarities between Manganism and Parkinson's Disease, and the fact that manganese accumulates in brain regions rich in dopaminergic neurons, it has long been suggested that the

neurotoxicity caused by manganese involves a disruption in dopaminergic neurotransmission[36-38].

Until recently, clinical interventions for Manganism have not been successful[39]. However in 2006, Jaing *et al.*[39] reported the first effective treatment of Manganism. Treatments with the drug p-aminosalicylic acid (PAS) reversed many of the symptoms of severe manganese intoxication in the patient. PAS is an anti-inflammatory drug which has been used to treat tuberculosis. They indicate that the exact mechanism of the drug action is unknown and that further studies of PAS in the treatment of manganese intoxication is necessary.

Bivalves and other marine invertebrates are often used in metal environmental toxicology studies because their tissues readily accumulate trace metals to concentrations that are usually much higher on a wet weight basis than what is present in the surrounding seawater[40-42]. Numerous reports have been made on the bioaccumulation of various heavy metals in the eastern oyster, *Crassostrea virginica*, and other oyster species[43-49]. Dopamine, serotonin and other biogenic amines are present in the nervous tissue and gill of *C. virginica*[50]. The animal has a reciprocal dopaminergic and serotonergic innervation of the lateral ciliated cells of the gill, originating in the cerebral and visceral ganglia, which slow down and speed up the beating rates of the cilia, respectively[51].

The animal is a simple system with which to study the relationships among biogenic amines, neurotoxins and other chemicals which may interact with them. Our lab reported that *C. virginica* incubated in $MnCl_2$, readily accumulated manganese into its ganglia and tissues[52] and caused a reduction in the levels of dopamine in the oyster's cerebral ganglia, visceral ganglia and gill, while having no effects on levels of other biogenic amines, including serotonin, epinephrine and octopamine[53]. We also showed that manganese treatments impaired the animals dopaminergic innervation of the lateral ciliated cells of the gill, but did not impair the serotonin innervation[54].

In the present study we sought to use *C. virginica* as a model to study the effects of PAS treatments on the effects of manganese on a known dopaminergically innervated system.

Materials and Methods

Oysters were maintained in Instant Ocean® artificial seawater (ASW) obtained from Aquarium Systems Inc. (Mentor, OH). Dopamine, serotonin, norepinephrine, epinephrine, octopamine and 1-octanesulfonic acid (sodium salt, SigmaUltra) were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents including manganese chloride ($MnCl_2 \cdot 4H_2O$, ASC grade) were obtained from Fisher Scientific (Pittsburgh, PA).

Adult *C. virginica* of approximately 80 mm shell length were obtained from Frank M. Flower and Sons Oyster Farm in Oyster Bay, NY. They were maintained in the lab for up to two weeks in temperature-regulated aquaria in ASW at 16 - 18º C, specific gravity of 1.024 ± 0.001, salinity of 31.9 ppt, and pH of 7.2 ± 0.2. Each animal was tested for health prior to experimentation by the resistance it offered to being opened. Animals that fully closed in response to tactile stimulation and required at least moderate hand pressure to being opened were used for the experiments. In order to ensure that each oyster would receive equal exposure to manganese during the experiment and not just close up, healthy specimens were shucked by removing their right shell before being placed into individual temperature-controlled aerated containers of ASW for 3 days in the presence of up to 1.0 mM of manganese and PAS. Control animals were similarly prepared without exposure to added manganese and PAS. Both control and experimentally treated animals tolerated the 3-day treatment well. Survival was excellent, there were no fatalities, and only animals with visible signs of heart pumping were used in subsequent experiments.

Endogenous serotonin and dopamine levels of the cerebral and visceral ganglia, as well as the gill were measured using high performance liquid chromatography (HPLC) with fluorescence detection based on the method of Fotopoulou and Ioannou[55]. Animals were treated for three days with or without manganese and PAS after which time each animal's cerebral ganglia, visceral ganglia and gill were excised and prepared for HPLC measurements of amine levels. The dissected tissues were homogenized with a Brinkman Polytron homogenizer with Omni International disposable probe tips in 0.4 M HCl. They were centrifuged at 12,000 x g for 20 minutes and then vacuum filtered through a 0.24 micron filter. Aliquots (20 µl) of the samples were injected into a Beckman System Gold 126/168 HPLC system fitted with a Phenomenex-Gemini (Torrance, CA) 5µ C18 reverse phase, ion pairing column with a guard column. The mobile phase was 50 mM acetate buffer (pH 4.7) containing 1-octanesulfonic acid (1.1 mM) and EDTA (0.11

mM), mixed with methanol (85:15 v/v). All reagents were HPLC grade. The flow rate was 2 ml/min in isocratic mode. A Jasco FP 2020 Plus Spectrofluorometer was used for detection of native fluorescence (280 nm excitation, 320 nm emission) and was fitted with a 16 µl flow cell. HPLC results are reported as ng/g wet weight for gill and ng/ganglion for the ganglia. Statistical analysis comparing dopamine and serotonin levels in gill and ganglia of treated animals to the controls was determined by a Two-way ANOVA.

Results

Serotonin and dopamine are able to be separated and quantified using HPLC with fluorescence detection. Chromatographs of the amine peaks for standards and a cerebral ganglion are shown in Figures 1a,b. The sensitivity is in the high picogram levels (Fig. 2).

Endogenous dopamine levels in the cerebral and visceral ganglia of untreated animals are in the range of 20 ng/ganglion and 200 ng/g wet weight for the gill. Treating animals for 3 days with 500 µM and 1 mM of manganese significantly reduced dopamine levels in the ganglia and gills (Fig. 3). Serotonin levels were not significantly changed in any of the tissues as a result of either treatment (Fig. 4). The dopamine levels in the ganglia and gill of animals which were co-treated with manganese and PAS were not significantly reduced when 500 µM of PAS was administered with the 500 µM of manganese. However, when 1 mM of PAS was administered with 1 mM of manganese, the dopamine levels in the ganglia and gill were significantly reduced as compared to the untreated controls (Fig. 3). The treatment with manganese and PAS caused no changes in serotonin levels in the ganglia and gill (Fig. 4).

Discussion

An earlier study showed that a 3-day treatment of *C. virginica* with manganese disrupted the oyster's dopaminergic, cilio-inhibitory mechanism, while not impairing the serotonergic cilio-excitatory mechanism[54]. The study also showed that manganese treatments lowered endogenous dopamine, but not serotonin levels, in the gill, cerebral ganglia and visceral ganglia of manganese-exposed animals compared to controls. Taken together, the results indicate the specificity of manganese toxicity on the animal's dopaminergic system. The results of that study suggest that the observed physiological deficits resulting from manganese treatments could be due to several contributing factors, most likely a combination of effects involving reduced levels of neuronal dopamine, possible destruction or damage of dopamine neurons, reduction of terminally released dopamine, and impaired post-synaptic responses to terminally released of dopamine.

It the present study, manganese treatments decreased dopamine levels in the ganglia and gill, but not serotonin levels and PAS protected

Figure 1a. HPLC Chromatogram

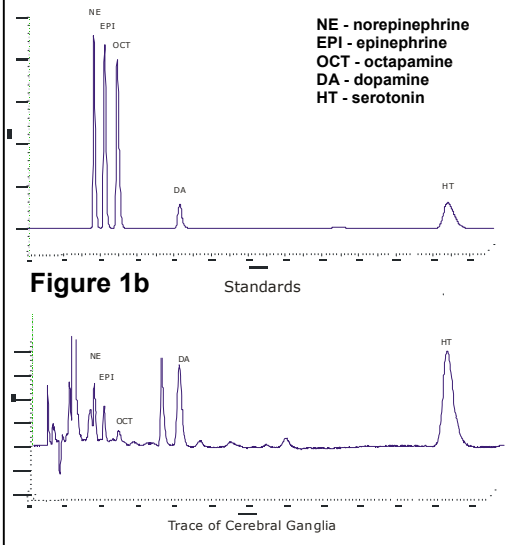


Figure 1b

Figure 1a. Chromatogram of amine standards.
Figure 1b. Chromatogram of cerebral ganglia

Figure 2. Amine Standards

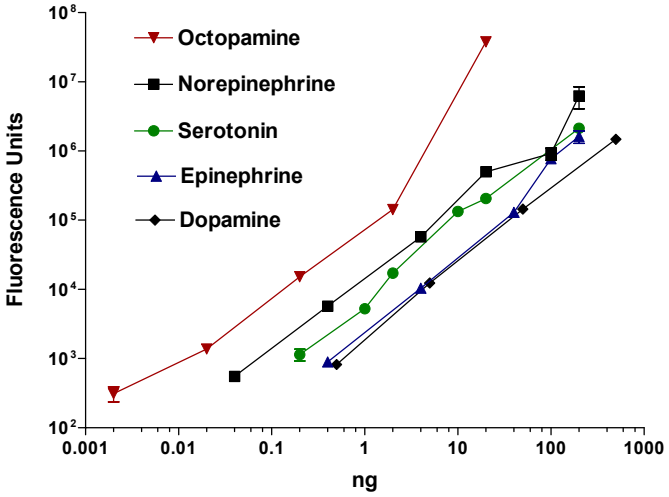
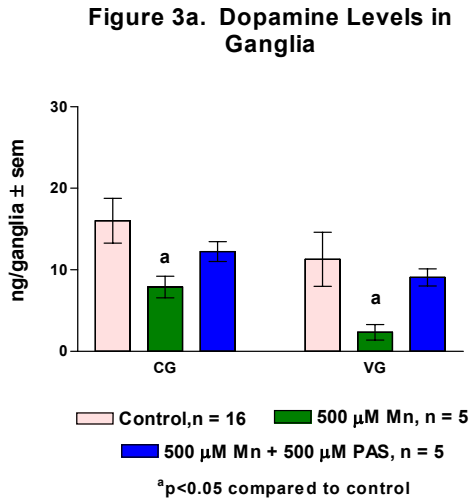
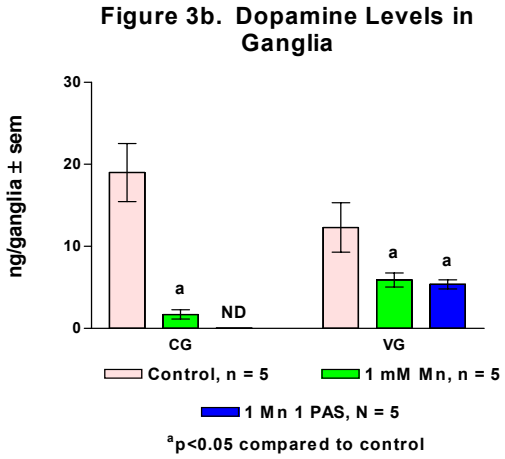
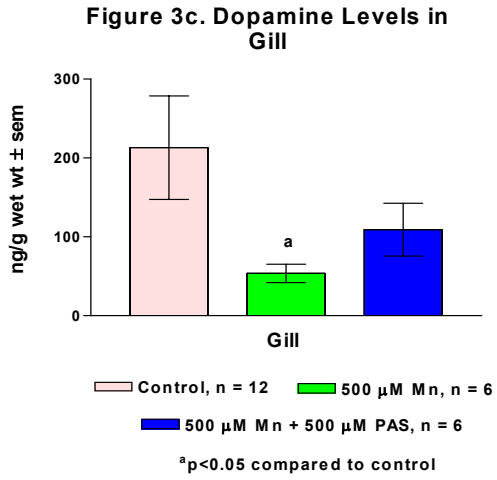


Figure 2. Graph showing relative fluorescence of amine standards at excitation wavelength of 280 nm and emission wavelength of 320 nm.







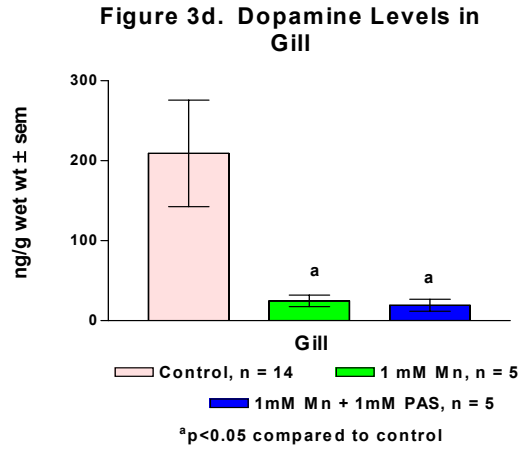
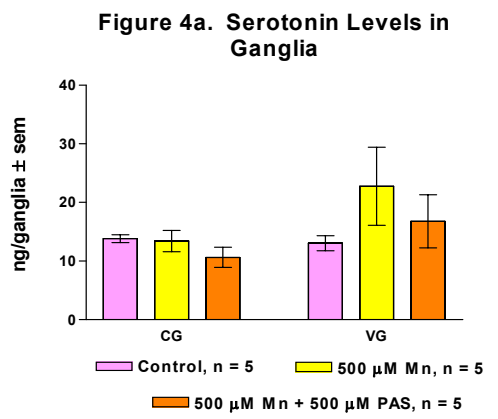


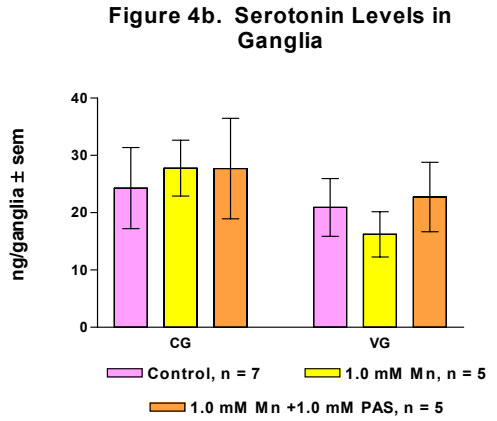
Figure 3a. dopamine levels (ng/ganglion ± sem) in the cerebral (CG) and visceral ganglia (VG) of animals treated with 500 µM of manganese (Mn), and 500 µM of Mn and PAS (p-aminosalicylic acid). **Figure 3b.** dopamine levels CG and VG of animals treated with 1 mM of Mn, and 1 mM of Mn and PAS. **Figure 3c.** dopamine levels (ng/g wet weight ± sem) in the gill of animals treated with 500 µM of Mn, and 500 µM of Mn and PAS. **Figure 3d.** dopamine levels in the gill of animals treated with 1 mM of Mn, and 1 mM of Mn and PAS. Statistical significance comparing treated animals to untreated controls was determined by a Two way ANOVA.

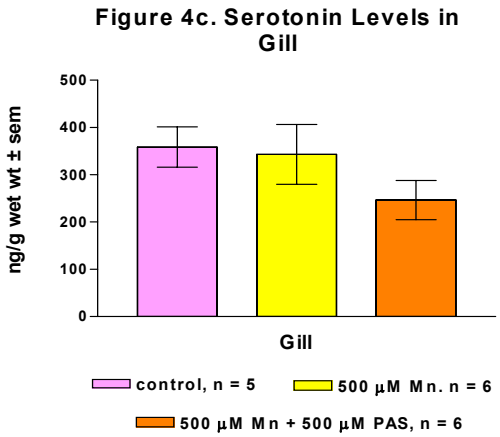
against the effects of the low dose of manganese on reducing dopamine levels in ganglia and gill.

Manganese is a trace element in animal systems required for normal carbohydrate, lipid, amino acid and protein metabolism, as well as a required cofactor for various antioxidant enzymes such as mitochondrial superoxide dismutase[1,56]. However when in excess, manganese is cytotoxic and has been shown to raise levels of reactive oxygen species[57,58], deplete glutathione[59], impair energy metabolism[60,61], and cause oxidation of catecholamine and other biological chemicals[62]. The prooxidant character of excess manganese and the fact that metal accumulates in dopamine-rich areas of the brain strongly suggests that manganese toxicity is causing further oxidative stress on an already stressed dopaminergic system[56,63-66].

While the cellular and molecular mechanism of manganese toxicity remains unclear, several lines of evidence suggest that exposure to manganese or manganese containing compounds induces oxidative stress-mediated dopaminergic cell death[67-69] which is in agreement with current theories on oxidative stress as a mediator of neuronal death in Parkinson's disease and other neurodegenerative diseases[70-74].







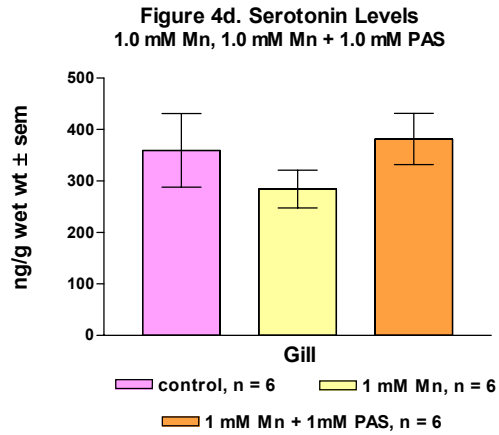


Figure 4a. serotonin levels in the CG and VG of animals treated with 500 µM of manganese (Mn), and 500 µM of Mn and PAS (p-aminosalicylic acid). **Figure 4b.** serotonin levels CG and VG of animals treated with 1 mM of Mn, and 1 mM of Mn and PAS. **Figure 4c.** serotonin levels (ng/g wet weight ± sem) in the gill of animals treated with 500 µM of Mn, and 500 µM of Mn and PAS. **Figure 4d.** serotonin levels in the gill of animals treated with 1 mM of Mn, and 1 mM of Mn and PAS. Statistical significance comparing treated animals to untreated controls was determined by a Two way ANOVA.

Dopaminergic neurons and dopamine-rich areas of the brain are particularly vulnerable to oxidative stress, because the enzymatic and non-enzymatic metabolism of dopamine can generate reactive oxygen species and various neurotoxic catecholamine metabolites such as 6-hydroxydopamine[73,75-78]. In a related bivalve, *Mytilus edulis*, a previous study showed that treatments with 6-hydroxydopamine caused a reduction in the animal's ganglionic levels of dopamine[79]. A recent study using transgenic mice provided *in vivo* evidence that chronic exposure to unregulated cytosolic dopamine alone was sufficient to cause neurodegeneration in striatal neurons and resulting motor dysfunction[80].

Jiang *et al.*[39] speculated that the mechanism of action of PAS in alleviating Manganism may be due to a chelating ability of PAS or that the salicylic acid moiety in PAS, which possesses an antiinflammatory effect, may contribute to therapeutic effectiveness of PAS in treatment of neurodegenerative Manganism. Other studies have suggested that nonsteroidal anti-inflammatory drugs, including sodium salicylic acid, may have neuroprotective benefit, because the inflammatory processes have been shown to play a role in the pathogenesis of neurodegenerative diseases such as Alzheimer disease, Parkinson's disease, and amyotrophic lateral sclerosis[81,82].

The present study demonstrates that the gill and ganglia preparations of *C. virginica* can be used to investigate mechanisms that underlie manganese neurotoxcity, and may also serve as a model in the pharmacological study of drugs to treat or prevent Manganism and perhaps other dopaminergic cell disorders.

Acknowledgments

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