2010-01-01

Experimental Study Of The Effects Of Green Tea

Alejandra Avila
University of Texas at El Paso, aavila5@miners.utep.edu

EXPERIMENTAL STUDY OF THE EFFECTS OF GREEN TEA
ON IMPROVING THE OUTCOMES OF BALB/c MICE INFECTED
WITH LEISHMANIA MEXICANA.

ALEJANDRA AVILA, B.S.

Department of Public Health Sciences

APPROVED :

_____
Rodrigo X. Armijos, M.D.,Sc.D. Chair

_____
Mary Margaret Weigel, Ph.D.

_____
Joaquin Rodrigo Garcia, Ph.D.

_____
Patricia D.Witherspoon, Ph.D.
Dean of the Graduate School

EXPERIMENTAL STUDY OF THE EFFECTS OF GREEN TEA ON IMPROVING THE OUTCOMES OF BALB/c MICE INFECTED WITH LEISHMANIA MEXICANA.

by

ALEJANDRA AVILA, BS

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER SCIENCE

Department of Public Health Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

December 2009

ACKNOWLEDGMENTS

I would like to gratefully acknowledge the help and support provided to me by my professor and advisor Dr. Rodrigo X. Armijos, because his invaluable support guided me and encouraged me to work hard and always to believe in myself. Also, I would like to extend my appreciation to the members of my committee, Dr. M. Margaret Weigel and Dr. Joaquin Rodrigo for their support and guidance in this process, thank you for your time and expert contribution. Also, I would like to thank all faculty members of the Health Promotion program because with their input and support during my studies help me to accomplish my goals and encourage me to keep on going in my professional career. Also, I would like to thank my classmates for their excellent technical assistance in the process of collecting data for this thesis, thank you Ximena Burgos and Laura Alvarez. I would also like to thank Mr. and Mrs. Janacek for their scholarship award which provided financial support to me during the pursuit of my M.S. degree.

Finally, I would like to thank my husband Omar for the support he gave me everyday, to my son this is all for you, and to my family, and close friends for their support and solidarity, without their love and company this achievement would not be possible.

Thank you very much.

ABSTRACT

Background. Leishmaniasis is a parasitic disease caused by an intracellular parasite belonging to the genus *Leishmania*. The cutaneous form of the disease causes often significant disfigurement, accelerates progression to clinical AIDS and tuberculosis, and is associated with a number of adverse economic, psychosocial, and nutritional consequences. An estimated 350 million persons are at risk for this globally distributed disease and 1.5-2 million new cases occur annually. Leishmaniasis is endemic in 88 countries on five continents. It is distributed throughout most of the Americas ranging from southern Texas to northern Argentina. It is also regarded as a threat to the readiness of U.S armed forces and civilian contractors deployed in Afghanistan, Iraq, and Kuwait. The sole first-line drug available for treating leishmaniasis is based on a heavy metal, antimony. It is associated with toxic side effects, is difficult to administer, is costly, and drug resistance is becoming increasingly common. The few available second-line drugs also are costly and have adverse side effects. In addition, there is no intervention available which can prevent the often extensive tissue destruction in patients caused by oxygen radicals generated during the immune response. The WHO has designated the development of new therapeutic alternatives for leishmaniasis as an important priority. Emerging evidence suggests that plant polyphenols such as those found in green tea may have beneficial health effects. Green tea contains a group of antioxidant scavengers (polyphenols) which have the ability to eliminate oxygen-free radicals that originate during inflammatory events. Green tea also is rich in ethylamine, which is recognized directly by γδ T lymphocytes and confers important γδ T-cell protective immune response during the early stage of certain infections (e.g., malaria, TB, herpes).

Objectives and Hypothesis. The major objective of the experimental study was to examine the potential beneficial effect of green tea (whole extract) as treatment for murine cutaneous leishmaniasis

caused by *Leishmania mexicana*. The *a priori* working hypothesis was that the use of green tea for the treatment of cutaneous leishmaniasis may provide some degree of protection from the deleterious effects of oxygen radicals originating during phagocytosis and thus improve clinical response during the early stage of infection.

Methods. 72 *Leishmania*-susceptible BALB/c mice were randomized to one of two groups. The experimental group (EG) with 36 mice drank green tea (1-1.2 mg/ml concentration) *ad libitum* and the control group (CG) of 36 mice drank tap water *ad libitum* for 14 weeks. During treatment week 4, mice were challenged by footpad injections of $1x10^6$ *L. mexicana* promastigotes. Infection progression was followed-up by measuring weekly footpad thickness. Every two weeks, 5 mice from each group were euthanized and their tissues harvested for immunological and parasitological studies. Peritoneal macrophage cultures were infected with *Leishmania* promastigotes to assess their microbicidal activity. Spleen lymphocytes were stimulated with *Leishamia* antigen to determine stimulation index, a cellular immune response indicator. Inflammatory cytokines were quantified by QRT-PCR. In addition, γδ T lymphocyte subpopulations in spleen leukocytes were assessed using flow cytometry.

Results. The average intake of green tea or water was estimated at ~9.17 ml/mice/day. The results indicated that the EG mice had reduced lesion size compared to CG mice ($\bar{x}$= 0.027 mm ± 0.198 vs. 0.09 mm ± 0.278). This pattern was evident by post-challenge week 8. However, by week 11, lesion size was similar in both groups. The *In vitro* analysis of infected peritoneal macrophages indicated that EG mice had fewer *Leishmania* parasites than CG mice when exposed to 5 ul ($\bar{x}$=17,877.33 ± 5578.16 vs. 20,466 ± 4029.95) or 2.5µl ($\bar{x}$=18,744 ± 11,092.58 vs. 22,179 ± 14,287.41) green tea

concentrations. The higher microbicidal activity also was evident even without any green tea ($\bar{x}$= 16405.88 ± 3705.21 vs. 25,757 ± 12,103.23). The lymphocyte proliferation assay results indicated that spleen lymphocytes from EG mice had a higher stimulation index when compared with those from CG mice ($\bar{x}$=5.73 vs 0.94).

Conclusions. Green tea increased cellular immune response and microbicidal macrophage activity. It resulted in a significant deceleration of infection progression until the week 8th post challenge. The results suggest that green tea has possible potential as an antileishmaniasis adjunctive therapy. Further studies are needed to identify specific compounds in green tea that possess apparent anti-*Leishmania* activity.

TABLE OF CONTENTS

LIST OF FIGURES

BACKGROUND AND SIGNIFICANCE

Epidemiology of leishmaniasis

Leishmaniasis is a parasitic disease of global public health importance. The World Health Organization (WHO) estimates that approximately 350 million persons around the world are at-risk for developing the disease, 12 million people are currently infected, and 1.5-2 million new cases occur each year (WHO, 2007). Leishmaniasis is endemic in 88 countries on five continents (WHO, 2007). It is present in Mexico, the southern tip of Texas and throughout the rest of the Americas region with the exception of Canada, Uruguay, and Chile (ibid). The incidence of the disease has been steadily increasing in many regions over the past several decades. One reason for this is the large increase in the number of persons with reduced immunocompetence due to HIV/AIDS (ibid). Other reasons include global climate change and deforestation of primary rainforest in developing countries which promotes host breeding of the sand fly vector leading to increased infection risk for endemic human population. Increased overseas travel and military deployment to endemic areas have also played a role in the increased disease prevalence. Leishmaniasis is considered a significant threat to the military readiness of coalition forces and civilian contractors serving in the Middle East. The Center for Disease Control (CDC) has reported in excess of 600 confirmed cases of the cutaneous form of leishmaniasis (CL) in U.S. soldiers deployed to Afghanistan, Iraq, and Kuwait, with many more cases suspected (CDC, 2005).

Leishmania parasite and its life cycle

The trypanosome parasite of the genus *leishmania* is the etiological agent for several of disease manifestations, collectively known as leishmaniasis (WHO, 1997). The infection of the human host is caused by 21 of 30 species that infect mammals. These include the *L. donovani*

complex with 3 species (*L. donovani*, *L. infantum*, and *L. chagasi*); the *L. mexicana* complex with 3 main species (*L. mexicana*, *L. amazonensis*, and *L. venezuelensis*); *L. tropica*; *L. major*; *L. aethiopica*; and the subgenus *Viannia* with 4 main species (*L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) panamensis*, and *L. (V.) peruviana*),(CDC, 2009). The different species are morphologically indistinguishable but can be differentiated by isoenzyme analysis, molecular methods, or monoclonal antibodies (CDC, 2009).

Leishmania cells have two morphological forms. The promastigote is extracellular with an anterior flagellum in the insect host, measures approximately 10 to 15 μm long, lives in the vector midgut. The amastigote form is intracellular organism without any flagella in the vertebrate host, they measure between 2 to 5 μm diameters (Ryan et al., 1990).

The life cycle begins when a parasitized female sandfly (*Phebotomus* spp or *Lutzomyia* spp) seek a bloodmeal. They become infected if they suck the blood of an infected human or wild mammal. Amastigotes become transformed into promastigotes in the sandfly gut and replicate. At a subsequent bloodmeal, promastigotes are injected into the skin of new host to complete the cycle (WHO/TDR, 2004).

Immunology of Leishmaniasis in Mammalian Host

To develop a successful parasitic relationship with the host, the *Leishmania* parasite must evade both the host innate and adaptive immune responses. Soon after *Leishmania* promastigotes enter the human body, they are phagocytosed by local macrophages. Once inside the phagolysosomal vesicle, *Leishmania* parasites are resistant to proteolysis and degredation. In addition, *Leishmania* avoids the immune system by remaining inside the macrophage (Bogdan and Rollinghoff, 1997). Murine leishmaniasis mimics cutaneous leishmanisis and viceral leishmaniasis forms from human leishmaniasis (Olivier et al., 2005). The level of animal

resistance or susceptibility depends upon the differential development of $CD4^+$ Th1 and Th2 cells. Healing is associated with efficient Th1 immune response mediated by increased levels of IL-2, IFN-γ, TNF-α, and IL-12, which enhance the anti-leishmanicidal defense mechanisms in the macrophages. The Th1 subpopulation plays an important role in cellular immune responses against intracellular pathogens by activating macrophages and other cell subpopulations leading to the intracellular killing of *Leishmania* sp. (Liew et al., 1999) Conversely, Th2 cytokines (IL-4, IL-5, and IL-13) are associated with disease chronicity. Recent studies have been shown that high levels of IL-10, an anti-inflammatory cytokine produced by T regulatory cells (Treg), suppress Th1 immune response in magnitude or function, this induce macrophage deactivation and thus promotes intracellular parasite proliferation. In contrast humoral immunity or antibody mediated immune response has been shown to be ineffective against leishmaniasis (Reiner and Locksley, 1995).

The γδT lymphocytes are important feature of the innate and adaptive immune systems. Recent studies have reported that the innate features and functions of these cells is very important such as the role of germ line elements of the T cell receptor for ligand recognition and segregation into functionally specialized cell populations in correlation with T cell receptor variable gene or protein expression. These cells attack the infection in the early stages. They also interact with cells of the innate system at many levels and the latest addition, their ability to present antigen. Thus, at present, much evidence suggests that γδT cells function in an innate manner, although they are arguably the most complex and advanced cellular representatives of the innate immune system (Born et al., 2006).

Clinical Forms of Human Leishmaniasis

Leishmaniasis has four major clinical manifestations. These depend upon the infective

parasite species involved and host immune response. Cutaneous leishmaniasis (CL) appears as a non-healing ulcer that can last anywhere from six months to around several years if left untreated. Diffuse cutaneous leishmaniasis is characterized by incurable infiltrated skin lesions. Mucocutaneous leishmaniasis (MCL) presents as ulcerative or granulomatous lesions of the nasal, oral, and pharyngeal linings. This form usually occurs after or concurrent with CL. Viceral leishmaniasis (VL) is the most severe form of leishmaniasis. Untreated VL causes chronic infection of the liver, spleen and bone marrow. Untreated VL is associated with 95% mortality (Carrion, 2006).

Leishmaniasis Treatment

There are currently no approved vaccines for any form of leishmaniasis. The complex epidemiological characteristics of leishmanisis and their transmission have limited the success of control efforts in endemic areas (Armijos et al., 2004). Parenteral antimonial drugs have been used as the gold standard for the treatment of leishmaniasis for many decades. However they require close medical supervision due to their well-documented cardiovascular, hepatic and renal toxicity (Murray, 2004). Antimonial drugs are contraindicated in individuals with drug sensitivity, certain chronic conditions, elderly patients, very young children, pregnant and breastfeeding women (Armijos et al, 2004), further limiting their usefulness.

Antimonial drugs are still classified as experimental in the U.S. Special permission is require for their use because they do not have Federal Drug Agency (FDA) approval (Murray, 2004). In many developing countries, antimonial drugs used to treat leishmaniasis are difficult to find and prohibitively expensive since a complete treatment course may cost $120 or more per person. This financial burden is magnified in rural endemic areas where multiple family members often require treatment at the same time. Due to these and other barriers, relatively few

individuals with CL ever receive any antimonial drug treatment and the majority of those do not complete treatment (Weigel et al., 2001; 1995; 1994). Second and third-line treatments such as amphotericin B, paramomycin and pentamidine are sometimes effective against leishmaniasis but also have severe side effects (Calvopina et al., 2004; Armijos et al., 2004). A recent study using intra muscular administration of paromomycin resulted in a cure of approximately 95% of VL cases, including in patients who had failed prior antimonial treatment (Murray, 2004). Miltefosine is also reported to be efficacious against VL but its activity appears to be limited against New World CL (Murray, 2004). The Tropical Disease, Special Programme for Research and Training (TDR) of the World Health Organization has classified the development of vaccines and alternative treatments as having high priority (TDR, 2004). One of the alternative treatments being developed by scientist for leishmanias involves natural plant products (TDR, 2004).

Biological Properties of Green Tea

Green tea a natural plant product with a long and valued history in traditional Chinese medicine (Cooper et al., 2005). Green tea is the second most consumed drink after water (Ahmad, 1997). Emerging evidence from epidemiological studies suggests that green tea may have beneficial health effects in humans. For Example, frequent tea consumption is associated with a decreased risk for cognitive impairment (Kuriyama, 2006). Its also has been linked with reduced mortality due to all causes and specifically cardiovascular disease mortality. However no significant association was found between green tea consumption and the risk of cancer (Nakachi et al., 2000).

Tea is produced from the leaves of the *Camellia sinensis* plant. What makes each tea different is the way it is processed. Black tea, one of the most common teas, is mostly grown in

southern Asia and Africa. In contrast to black tea is made by allowing tea leaves to completely ferment while drying. Black tea has a strong flavor and contains more caffeine than less fermented teas. Tea leaves go through only a minimal amount of fermentation to make green tea. This is accomplished either with steam (traditional Japanese method) or by drying in hot pans, (Chinese method). Gunpowder tea is a type of green tea in which each leaf is rolled into a small pellet. However this method is usually done only with tea leaves of very high quality (Princen, 1998). Emerging evidence indicates that green tea is rich in a variety of beneficial chemical substances including polyphenols, essential oils, methylxantines, vitamins and amino acids (Yamamoto, 1997).

Green tea may also have antioxidant benefits. This has important implications for the control of leishmaniasis because the generation of oxygen radicals is an important host defense mechanism against intracellular and extracellular microorganisms (Frei and Higdon, 2003). Oxygen radicals denature cellular proteins and lipids, and cause DNA fragmentation on target microorganisms as well as up the surrounding host tissue. Chronic infectious diseases such as leishmaniasis, leprosy, Chagas disease and tuberculosis are characterized by long-term inflammation associated with continuous oxygen radical generation, among other defense mechanisms, as the host immune system tries to control the infection. This type of defense mechanism generates significant tissue destruction in the host (Frei and Higdon, 2003).

The catechins which belong to the flavonol family are the predominant polyphenols in green tea (Lee et al., 2002). Catechins have been shown to possess strong antioxidant properties *in vitro*, inhibiting the peroxidation of phospholipids and both Low density lipoprotein copper-ion and cell-mediated oxidation (Scalbert et al., 2002). Less is known about the more complex polyphenols, such as theaflavins that are in black tea, but they have also been reported to be also

antioxidants (Lambert et al., 2007). In addition to the antioxidative properties of tea, epidemiological studies have demonstrated an association between the frequent consumption of green and black teas and reduced plasma cholesterol and triglyceride levels. They may also explain the reduced cardiovascular mortality associated with tea consumption (Princen et al., 1998).

Most of these compounds possess medically important biological properties such as antioxidant and free radical scavenging, anti-inflammatory, antimicrobial, anticancer, hypolipidic, and immune regulatory activities (Engwerda et al., 2004). The most highly concentrated polyphenols found in green tea which have significant antioxidant activity are epigallocatechim-3-gallate, epigallocatechim, epicatechim and epicatechin gallate (Higdon and Frei, 2003). These have been reported to abate various signaling pathways important in pathological processes such as inducing of dose-dependent apoptosis and G_0—G_1 phase arrest in cancer cell (Ahmad et al 1997). Among other activities, they also inhibit progression of experimental caries caused by mutant *Streptococci* in rats (Otake et al. 1991), protect against experimental infection with *V. cholerae* 01 (Toda et al 1992). These compounds also have antiprotozoal lytic activity for bloodstream (trypomastigote) and intracellular (amastigote) *T. cruzi* forms (Paveto, et al. 2004). Furthermore, different degrees of *In vivo* and *In vitro* activity against *Leishmania donovani* (the causative agent of VL), *T. brucei* and *T. cruzi,* have been reported to characterize some synthetic flavonoids and their analogues. For this reason, these potential antiprotozoal agents require further investigation (Tasdemir et al., 2006).

In addition to its important anti-microbial polyphenolic activities, green tea also is rich in ethylamine, a product of L-theanine hydrolysis that occurs in the intestines and liver. Alkylamides are also present in microorganisms (bacteria, parasite, and virus) and tumor cells

antigens. In humans, ethylamine antigen is recognized directly by γδ T lymphocyte (Vγ2Vδ2) T-cells in a TCR-dependent manner. It induces their activation, an important defense mechanism that occurs during innate immune response (Thompson, 2006). In mice, it has been shown to confer important γδT-cell protective immunity during the early stage of certain infections such as malaria caused by *Plasmodium yoelii* in liver and blood (Moriya et al., 1994) and pre-erythrocytic parasite stages (McKenna et al., 2000). In addition, it appears to confer protective immunity in murine leishmaniasis caused by *L. major*, where depletion of γδT-cell population is associated with fast rapid lesion growth (Rosat et al., 1993), and increases severity of tuberculosis (Laden et al 1995). Likewise, in primary infections caused by *Listeria monocytogenes* (Hiromatsu et al., 1992), ethylamine antigen plays an important function via regulation of NK cells which predominate during early host anti intracellular infection (Ladel et al., 1996), it also as plays a critical role in the early inflammatory response to *Bordetella pertussis* infection (Zachariadis et al., 2006), and in encephalitis cause by Herpes virus-1 (Sciammas et al., 1994).

Cao and Colleges (2007) have reported that green tea can modulate TTP mRNA levels in animals. They have suggested that a post-transcriptional mechanism through TTP (Tristetraprolin) could partially account for the anti-inflammatory properties of green tea. Their study findings also suggest that drinking adequate amounts of green tea may play a role in the prevention of inflammation-related diseases (Cao et al., 2007).

Monobe and associates (2008) also have reported that the immune stimulating activity of crude polysaccharide from immature tea leaves was higher than that of TPS (immune stimulating activity of tea polysaccharide) one of the main components from the green tea extract. They concluded that the catechin-polysaccharide complex in immature tea leaves appears to be a

potential immune stimulator (Monobe, 2008). In Addition, green tea and its active components have been shown interfere with signal transduction pathways necessary for *Leishmania* survival (Feily, 2009).

STUDY RATIONALE

Studies are needed to investigate the effects of green tea as an alternative anti-*Leishmania* treatment compare to the expensive and toxic treatments currently available. To date, there is no safe and effective alternative treatment for CL. In addition, there is no intervention available to prevent the often extensive tissue destruction in patients with leishmaniasis. Green tea was chosen because it contains the larger amount of compounds with anti leishmanial activity as well as antioxidant properties (Frei, 2003, Mendoca-Filho, 2004).

STUDY HYPOTHESIS AND OBJECTIVES

Study Objectives

The overall objective of the proposed experimental study was to investigate the anti-leishmanial activity of green tea in murine cutaneous leishmaniasis model caused by *Leishmania mexicana*.

Specifc Aim 1. To characterize the innate and cellular immune response modulated by green tea treatment against a murine leishmaniasis animal model. Immunological assays were carried out to examine the immune response in murine cutaneous leishmaniasis.

Specific Aim 2. To determine the association between disease progression and cellular immune response in the EG and CG mice. To test this, footpad lesions were measure periodically to compare with the level of the immune response at different time period.

Hypothesis

Is hypothesized that the antioxidant compounds present in green tea act to induce immunostimulating activity against murine leishmanisis caused by *Leishmania mexicana*.

METHODOLOGY

Experimental Animals. The experimental design study used 72, six-week old female BALB/c mice. This mouse (Harlan Laboratories) strain is well documented to be susceptible to infection with *Leishmania Mexicana (*Padigel*, 2003).* The green tea group (n=36) drank the green tea *ad libitum* and the control group (n=36), received tap water *ad libitum*. These were the only sources of liquids for both groups also received regular diet and were treated in exactly the same manner with respect to light cycles, bedding and other environmental variables.

Leishmania Parasite. *Leishmania mexicana* promastigotes (L4 strain) were cultured in RPMI 1040 media supplemented with 10% fetal bovine serum with alternate passes through previously activated U-937 human macrophages cell line to maintain virulence. The *Leishmania mexicana* strain was isolated from a leishmaniasis patient from Tabasco State, Mexico.

Experimental Treatment. The green tea beverage was consumed by the experimental mice group was prepared by adding 200ml of boiling water to a 2-gram bag of tea (Empacadora Therbal, Mexico DF) and steeping it for 10 minutes. This procedure yields a polyphenol concentration of 1-1.2 mg/ml using the Folin-Ciocalteu assay according to experiments perform by Dr. L. De la Rosa, (personal communication).

Infection Challenge and Lesion Measurement. Two weeks after the experimental group began reciving the green tea they were challenged subcutaneously in the right hind footpad with 40 µl of 5 x 10^4 live stationary phase *L. mexicana* promastigotes. The development of footpad lesions (i.e., swelling) at the infection site was measured and recorded once a week using a digital caliper. The measurements were expressed as the difference between the thickness of the infected versus the contra-lateral non-infected footpad on the same experimental animal

Serum Antioxidant Activity assay: Venous blood was drawn from the maxillary plexus of the

mice and collected in a vial with EDTA. After centrifugation, 24 µl of serum diluted 1:4 in distilled water and mixed with 180 µl of a solution consisting of acetic acid and sodium acetate buffer pH 3.6: 10 mM 2,4,6 tripyridyl-1,2,3 triazine/ 40 mM HCl/ 20 mM $FeCl_3$. Absorbance was measured at 595nm using a Victor^3 1420 multilabel counter (Perkin-Elmer), every 30 seconds during one hour period. To determine the concentration of reduced Fe_3 to Fe_2, the absorbance was substituted after 30 minutes of measurement on the calibration curve equation. The calibration curve was prepared adding known concentrations of $FeSO_4$ as an equivalent of Fe^{+2}(Benzie and Strain, 2005)

Immunological Measurements. Two weeks after the mice began drinking the green tea (experimental group) or water (control group), six animals per group were euthanized to obtain baseline data. The remaining animals were inoculated with *L. mexicana* parasites in their footpads as previously described. In order to compare cellular immune responses between the experimental and control groups, at post-parasite challenge day 3 and weeks 1, 3, 5, 7, 9 a total of five mice per group were sacrificed using CO_2 inhalation. These animals were used as tissue for the post-mortem immunological and parasitological studies. The mouse blood was collected in sterile vials. In addition, mouse spleen and regional lymph nodes were used as a source of T-cells for the immunological studies were deposited in sterile vials containing RPMI 1040 medium supplemented with 10% fetal bovine serum.

Cell Proliferation Assay. Spleens and lymph node cells from each study group mouse were removed aseptically to prepare a single cell suspension. The cells were washed with 0.9% ice-cold ammonium chloride to induce lysis of the erythrocytes. Leukocytes were re-suspended to a density of 2.5 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Soluble *Leishmania* antigen (4 g/ml) or ConA (2 µg/ml) was added in a final volume of 200 µl

well. Cells were incubated for three days at 37°C in an atmosphere containing 5% of CO_2. Proliferation was measured by incorporating of 1 μCi of ^{3}H-thymidine over the last 18h of the culture. The incorporation of ^{3}H-thymidine was measured by a liquid scintillation counter (Perkin-Elmer). The stimulation index was calculated as the ratio of stimulated/unstimulated incorporation of ^{3}H-thymidine.

Flow Cytometric Analysis of NK γδT-Cell Population. Lymphocytes (10^6 cells) from regional lymph nodes were incubated in 2% normal rabbit serum at 0°C for 20 minutes and normal mouse serum at 0°C for 15 minutes. Samples were incubated in the dark on ice for 20 minutes with 100 μl of the appropriate Armenian hamster anti-mouse γδT-TCR antibody conjugated to fluorescein isothiocyanate, Armenian hamster anti-mouse NK1.1 antibody conjugated to phycoerythrin, or Armenian hamster anti-mouse CD3 antibody conjugated with peridinin chlorophyll α protein (Becton Dickinson, PharMingen, San Diego, CA). Isotype control antibodies were used in control samples. Cells were washed twice with PBS and relative fluorescence intensities were determined on a 4-decade long scale by flow cytometric analysis using FACSCalibur instrumentation (Becton Dickinson, Mountain View, CA). Data were analyzed using CellQuest sofware (Becton Dickinson). This assay was performed in the Transplant Immunology Laboratory of Sierra Medical Center-El Paso.

Leishmanicidal Activity of Peritoneal Macrophages. Peritoneal macrophages were obtained immediately after euthanasia. Five ml of RPMI medium was injected in the peritoneal cavity and a medium containing peritoneal macrophages was aspirated, and deposited in a sterile vial placed on ice. Monocytes (3×10^5 cells) were cultured and activated in 24 well culture plate. After 48 hours, stationary phase growth *Leishmania* promastigotes was added at a ratio 14 parasites/cell. After 4h of incubation with 5% CO2 at 35 °C, the culture was washed twice with warm RPMI,

and green tea extract or PBS (control) was added. After 72 hours, the cultures were washed and the infected macrophages were removed using a rubber stick and cultured at 25 ^{0}C. After 54 hours, parasite growth was determined by adding 1 μCi [3H] thymidine to each well. The parasites were harvested 18h later and [3H] thymidine incorporation was measured with the liquid scintillation counter.

Sample Size and Power

There was 36 animals in each of the two study groups. The sample size was calculated if one assumes an α of 0.05, a 1-β of 0.80, equal variance in the genetically similar groups, and an estimated difference of 35-50% between the experimental and control groups in a 2-sided test. The calculation of sample size was using published sample size calculation tables (L Gordis, *Epidemiology*, 3rd ed., 2004).

Statistical Analysis

The data were entered in SPSS (Version 14). Means with their respective and standard deviations were calculated to compare the differences between the Experimental group and the Control group. Two-tailed Student's t-tests. For the cell proliferation assay, the stimulation index was calculated to compare the cell with antigen and the cells with concanavaline A.

RESULTS

Experimental Treatment

The green tea beverage was prepared at a concentration of ~1-1.2mg/ml. The average green tea or water intake was ~9.17 ml per mouse/day. The estimated mean daily oral intake of green tea ranged from 9.2 mg to 11.0mg/mouse. No side effects such as over sleeping, curling, low sociability were associated with the intake of green tea beverage during the study period.

Leishmanicidal Activity of Peritoneal Macrophages

As Figure 1 shows, the analysis of anti leishmanicidal activity of peritoneal phagocytic cells revealed that macrophages from EG mice significantly killed more parasites than the CG when 2.5 µl or 5 µl of green tea were added. This capacity was more evident at day 67 (Figure 3) post parasite challenge ($\bar{x}$=17877 ± 5579 parasites vs. 20,466 ± 4030 parasites) or 2.5µl ($\bar{x}$=18,744 ± 11093 parasites vs. 22,179 ± 14287 parasites) however there was no statistical significance (t=0.328; p=0.758). The highest microbicidal activity was more apparent in the absence of green tea (Figure 2) in the EG at day 67 ($\bar{x}$= 16,406 ± 3705 parasites vs. 25,758 ± 12,103 parasites) but there was no statistical significance (t=1.279; p=0.269).

Flow Cytometric Analysis of NK γδT-Cell Population

Figure 5 shows the results revealed that popliteal γT-cells from EG mice were increased at day 4th post parasite challenge compare to the CG ($\bar{x}$= 64.35 ± 6.54 cells vs 30.15 ± 26.27cells) but no statistical significance was found (t=1.719; p=0.184). The same situation was again evident when δT-cells were targeted ($\bar{x}$= 64.29 ± 6.54 cells vs 30.13 ± 26.25 cells). But not significant differences (t=1.719; p=0.184) between EG and CG were found on subsequent measurements (Figure 6)

Cell Proliferation Assay

Lymphocyte stimulation index assay was measure as an indicator of immune function. There was a higher stimulation index of spleen cells from the EG mice activated with *Leishmania* antigen compare to the CG at baseline (5.18 vs 0.55) however there was no statistical significance between the groups (t=-0.522; p= 0629), at day 4 there is also a difference between the groups with the stimulation index (6.57 vs 3.38) but no statistical significance (t=-1.833; p= 0.141), another difference is at day 67, the EG having a superior activation against the CG (3.03 vs 1.66) but do to the small sample size there was no statistical significance (t= -0.66; p= 0.545). The stimulation index was also higher in the EG mice when activation were induced with ConA at baseline (3.86 vs 1.58) but with low statistical significance (t=2.350; p= 0.078); and at day 67 (5.55 vs 0.94) however there was no statistical significance (t=1.316; p=0.258).

Infection Challenge and Lesion Measurement

Figure 7 shows that EG Mice had reduce lesion size at base line ($\bar{x}$=0.010mm ± 0.214 vs 0.078mm ± 0.056), at day 28 ($\bar{x}$= 0.027 mm ± 0.198 vs. 0.09 mm ± 0.278) there is a low statistical significance (t=-2.279; p= 0.033) until day 67 ($\bar{x}$=0.043mm ± 0.227 vs 0.094mm ± 0.206) with a statistical significance (t=-2.960; p= 0.007). However, by day 101, lesion size was similar in both groups ($\bar{x}$=0.164mm ± 0.147 vs 0.148mm ± 0.12).

DISCUSSION

The primary objective of this study was to determine whether green tea possessed effectiveness as a possible treatment for murine cutaneous leishmaniasis. The footpad infection results indicated that daily green tea in the 9.2-11.0mg range intake induced protection in mice until study day 67. This initial protection in terms of non apparent lesion development was also associated with an increased number of γδT cell subpopulations as well as a higher T cell proliferation index. These findings suggest that the stimulatory innate response mediated by γδT cells appeared to have protective effect at the early stage of *Leishmania* infection followed by an efficient T cell mediated immune response. However, by day 89 and later, the leishmaniasis lesions in both groups were comparable in size and growth rate. This indicates that green tea alone in not sufficient to completely control *Leishmania* infection, but could be useful as an adjunctive therapy in combination with standard treatment. Previous studies have reported that ethylamine, a product of green tea L-theamine hydrolysis in the intestines and liver is recognized directly by γδT cells in a TCR-dependent manner and induces their activation during early stages of the innate immune response (Moriya, 1994). The immune activation capability of green tea may not be antigen specific, because in addition to the increased antigen induced stimulation index, the T cell activated with ConA also shown increased proliferation index in the EG compare to the CG. Cell proliferation assay is a major technique for assessing the functional capacity of $CD4^+$ lymphocytes to respond to various stimuli (Lederman, 1998).

Leishmania is a well adapted obligated intracellular microorganism, able to infect, replicated and persist inside activated macrophages. Some of the answers to leishmaniasis control may be on determining molecular aspects of *Leishmania*-macrophage interaction. The

increased anti-leishmanicidal activity of EG peritoneal macrophages cultures confirmed the immunostimulatory effect of green tea when given in an average daily oral intake of ~9.17 ml per mice of ~1-1.2mg/ml. The leishmanicidal effect was increased by adding 2.5 or 5 mg of green tea to the infected macrophage cultures. This effect was evident even in EG macrophage cultures where no green tea was added. The previous *In vivo* exposure of macrophages to green tea was effective enough to later increase the mycrobicidal activity of macrophages infected with *Leishmania mexicana* promastigotes *In vitro* as has been described previously (Anand, 2005). These results corroborate previous studies suggesting that green tea polyphenols significantly increase macrophage activation and phagocytosis capability (Cao et al., 2007).This also suggested this can increase microbicidal activity in the early immune response (Yamamoto, 2004) and increase the leishmanicidal properties of macrophages (Mendoca-Filho, 2004).

In conclusion, early increase in γδT cells, increased lymphocyte proliferation index, and efficient leishmanicidal activity of macrophages were associated with reduced lesion size in green tea treated mice. In contrast, decreased γδT cell population and low proliferation index were associated with increased lesion size and disease progression as was seen at the end of this study. These findings suggest that green tea provides an initial immune boost but does not provide long lasting protection response against *Leishmania mexicana*

The strengths and limitations of this study should be considered when interpreting its results. One of the strengths was that there consistent pattern across the immunological assays regarding early infection protection induced by green tea. One limitations of the pilot study is the small sample size and low statistical power that may have affected the statistical results. The small sample size of the study was necessitate by regulations of animal IRB that prohibit using a large number of animals.

In conclusion, early increased of γδT cells, increased lymphocyte proliferation index, and efficient leishmanicidal activity of macrophages are associated with small lesion size in the EG mice. Likewise, decreased γδT cells population and low proliferation index are associated with increased lesion size and disease progression as was seen during later disease progression. These findings suggest that despite providing an early boost to immune response green tea does not long lasting protection response against *Leishmania Mexicana.* Therefore, green tea has a potential use as antileishmaniasis adjunctive therapy. Further studies are needed to identify specific compounds in green tea that possess apparent anti-*Leishmania* activity.

REFERENCES

Ahmad N, Feyes DK, Nieminen A, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3 gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Nat Cancer Inst*, 86(24)1881-1886, 1997.

Anand P, Kaul D, Sharma M. Green tea polyphenol inhibits *Mycobacterium* tuberculosis survival within human macrophages. *The international Journal of Biochemistry and Cell Biology,* 58:650-2, 2008

Armijos RX, Weigel MM, Calvopiña, M, Mancheno M. Comparison of the effectiveness of two topical paromomycin treatments versus meglumine antimoniate for New World cutaneous leishmaniasis. *Acta Tropica 2004;* 91:153-160.

Auger C, Al-Awwadi N, Bornet A, Rouanet JM. Catechins and procyanidis in Mediterranean diets. *Food Research Internacional,* 37(2004)233-245.

Bayer J, Gomer A, Demir Y, Amano H, Kish D, Fairchild R. Effects of green tea polyphenols on murine transplant-reactive T cell immunity. *Clinical Immunology.* 110 (2004) 100-108.

Benzie I, Strain J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of ‘‘Antioxidant Power’’: The FRAP Assay. *Analytical Biochemistry.* 239(0292):70–76, 1996.

Belkaid Y, Piccirillo C, Mendez S, Shevarch E. $CD4^{+}CD25^{+}$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature.* 420: 502-507, 2002.

Bogdan C, Rollinghoff M. The immune response to *Leishmania*: mechanisims of parasite control and evasion. *International Journal for parasitology.* 28:121-134, 1998.

Born W, Reardon C, O’Brien R. The function of γδT-Cells in innate immunity. *Current Opinion in Immnology.* 18:31-38, 2006.

Bukowski J, Morita C, Breener Michael. Human γδT-Cells Recognize Alkylamines Derived from Microbes, Edible Plants, and Tea: Implications for Innate Immunity. 11:57-65, 1999.

Calvopiña M, Guevara A, Armijos RX, Hasiguchi Y, Davidson R. Efficacy of Itraconazade in the Treatment of New World Mucocutaneous Leishmaniasis. *Int J Dermatology.* 43:659-663, 2004.

Cao H, Kelly M, Kari F, Dawson H, Urban J. Green tea increases anti-inflammatory tristetraprolin and decreases pro-inflammatory tumor necrosis factor mRNA levels in rats. *Jounal of inflammation.* 4:1-12, 2007.

Carrion J, Nieto A, Ibarra S, Iniesta V, Soto M. Immunohistological features of viceral leishmaniasis in BALB/c mice. *Parasite Inmmunology;* 28:173-183.2006.

Center for Disease Control. *Parasites and Health: Leishmanisi.*2008.

Cooper R, Morre J, Morre D. Medicinal Benefits of Green Tea: Part I. Review of Noncancer Health Benefits. *The Journal of Alternative and Complementary Medicine;* 11(3):521-528. 2005.

Cooper R, Morre J, Morre D. Medicinal Benefits of Green Tea: Part II. Review of Anticancer Properties. *The Journal of Alternative and Complementary Medicine;* 11(4):639-652. 2005.

Crespy V, Williamson G. A review of the health effect of green tea catechins in *In vivo* animals models. *American Society for Nutritional Sciences.* 1:3431S-3444S, 2004.

Desjeux P, The increase in risk factors for leishmaniasis worldwide. *Transaction of The Royal Society of Tropical Medicine and Hygiene,* 95:239-243. 2001.

Engwerda C, Ato M, Kaye P. Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. *TRENDS in Parasitilogy,*20(11):524-530.2004

Frei B, Higdon JV. Antioxidant Activity of Tea Polyphenols In Vivo: Evidence from Animal Studies. *American Society for Nutricional Sciences.* 1:3275S-3284S, 2003.

Graff J, Jutila MA. Differential regulation of CD11b on γδ T Cells and monocytes in response to unripe apple polyphenols. *Journal of Leukocyte Biology.* 82:603-607, 2007.

Hiromatsu K, Yasuobu Y, Matsuzaki G, Ohga S, Muramori K, Matzumoto J Nomoto K. a protective role of γδ T Cells in primary Infection with *Listeria monocytogenenes* in mice. *J Exp Med*, 175; 49-56, 1992.

Kamath A, Wang L, Das H, Li L, Reinhold V. Antigens in tea beverage prime human V γ2Vδ2 T cells in vitro and in vivo for memory and nonmemory antibacterial cytokine responses. *PNAS immunology.* 100(10):6009-6014, 2003.

Kharazmi A, Kemp K, Ismail A. T-cell response in human leishmanisis. *Immunology letters.* 65:105-108, 1999.

Kuriyama S, Hozawa A, Ohmori K, Shimazu T, Matsui T, Ebihara S, Awata S, Nagatomi R, Arai H, Tsuji I. Green tea consumption and cognitive function: a cross-sectional study from the Tsurugaya Project. *AmJ Clin Nutr.* 83:355– 61, 2006.

Ladel CH, Blum C, Kaufmann HE. Control of natural killer innate resistance against the intracellular pathogen *Listeria monocytogenes* by γδ T lymphocytes. *Infect Immun*, 64:1744-1749, 1996.

Lambert J, Sang S, Yang C. Biotransformation of Green Tea Polyphenols and the Biological Activities of Those Metabolites. *Molecular pharmaceutics.* 4 (6):819-825. 2007.

Lee MJ, Malikal P, Chen L, Meng X, Bondc F. Pharmacokinetics of tea catechins after ingestion of green tea and (--) –epigallocatechin-3-gallate by humans: Formation of different metabolites and individual variability. *Cancer Epidemiology. Biomarkers and Prevention.* 11:1025-1032, 2002.

Liew F, Xu D, Chan L. Immune effector mechanism in parasitic infections. *Immunology Letters.* 65:101-104, 1999.

McKenna K, Beignon A, Bhardwaj N. Plasmacytoid Dendritic Cells: Linking Innate and Adaptive Immunity. *Journal of Virology*, 79 (1):17-27, 2005.

Mendonca R, Rodrigues I, Alviano D, Santos A, Soares R. Leishmanicidal activity of polyphenols-rich extract from husk fiber of *Cocos nucifera* Linn (Palmae). *J. resmic.* 155: 136-143, 2004.

Monobe M, Ema K, Kato F, Maeda M. Immunostimulating Activity of a Crude Polysaccharide Derived from Green Tea (Camellia sinensis) Extract. *J. Agric. Food Chem.* 56:1423-1427, 2008.

Moriya T, Mombaerrts P, lefrancois L, Nussenzweig RS, Zavala F. γδ T Cells contribute to immunity against the liver stages of malaria in αβ T-Cells-deficient mice. *Proc Natl Acad Sci USA*, 91:345-349, 1994.

Murray H. Treatment of visceral leishmaniasis in 2004. *Am J. Trop. Med. Hyg.,* 71(6):787-794.2004.

Nakachi K, Matsuyama S, Miyake. Preventive effects of drinking green tea on cancer and cardiovascular disease: Epidemiological evidence for multiple targeting prevention. *BioFactors.* 13:49-54. 2000.

Otake S, Makimura M, Kuroki T, Nishihara Y, Hirasawa M. Anticaries effects of polyphenolic compounds from Japanese green tea. *Caries Res*. 25(6):438-43, 1991.

Olivier M, Gregory D, Forget G. Subversion Mechanisms by Which *Leishmania* Parasite Can Escape the Host Immune Response: a Signaling Point of View. *Clinical Microbiology Reviews.* 18(2):293-305, 2005.

Padigel U, Alexander J, Farrell J. The Role of Interleukin-10 in Susceptibility of BALB/c Mice to Infection with *Leishmania mexicana* and *Leishmania amazonensis.The Journal of Immunology.* 171:3705-3710, 2003.

Paveto C, Güida MC, Esteva MI, Martino V, Coussio J, Flawiá MM, Torres HN. Anti-Trypanosoma cruzi Activity of Green Tea (Camellia sinensis) Catechins. *Antimicrob Agents Chemother*; 48(1): 69-74, 2004.

Princen H, Duyvenvoorde W, Buytenhek R, Blonk C, Tijburg L, Langius J, Meinders A, Pijl A. No Effect of Consumption of Green and Black Tea on Plasma Lipid and Antioxidant Levels and on LDL Oxidation in Smokers. *Arteriosclerosis, Thrombosis, and Vascular Biology.*18:833-841, 1998.

Reiner SL, Locksley RM. The regulation of immunity to Leishmania major. Annu Rev Immunol. Review;13:151-77. 1995.

Rietveld A, Wiseman S. Antioxidant Effects of Tea: Evidence from Human Clinical Trials. *American Society for Nutritional Sciences.* 1:3285S-3292S, 2003.

Rosat JP, MacDonald HR, Louis JA. A role for gamma delta + T cells during experimental infection of mice with Leishmania major. *J Immunol*, 150: 550-555, 1993.

Russo DM, Armitage RJ, Barral M, Barral A, Grabstain K, Reed S. Antigen-Reactive γδ T Cells in Human Leishmanisis. *The Journal of Immunology.* 151(7): 3712-3718, 1993.

Ryan L, Vexenat A, Marsden P, Lainson R, Shaw J. The importance of rapid diagnostic of new cases of cutaneous leishmaniasis in pin-pointing the sandfly vector. *Transactions of The Royal Society of Tropical Medicine and Hygiene.* 84:786, 1990.

Scalbert A, Morand C, Manach C, Remesy C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother.*56:276-282, 2002.

Sciammas R, Kodukula P, Tang Q, Hendricks RL, Bluestone JA. T Cell Receptor-/ Cells Protect Mice from Herpes Simplex Virus Type 1-induced Lethal Encephalitis. *J Exp Med*. 185: 1969-1975, 1997.

Tasdemir D, Kaiser M, Brun R, Yardley V, Schmidt T, Tosun F. Antitrypanosomal and Antileishmanial Activities of Flavonoids and Their Analogues: In Vitro, In Vivo, Structure-Activity Relationship and Quantitative Structure-Activity Relationship Studies. *Antimicrobial Agents and Chemotherapy.* 50(4):1352-1364, 2006.

Thompson K, Rojas J, Rogers M. Alkylamines cause Vγ2Vδ2 T Cell Activation and Proliferation by Inhibiting The Mevalonate Pathway. *Immunobiology.* 107(2): 651-653, 2006.

Toda M, Okubo S, Ikigai H, Suzuki T, Suzuki Y, Hara Y, Shimamura T. The protective activity of tea catechins against experimental infection by *Vibrio cholerae* O1. *Microbiol Immunol*. 36(9):999-1001, 1992.

Weigel MM, Armijos RX, Racines J, et al. Cutaneous leishmaniasis in subtropical Ecuador: popular perceptions, knowledge, and treatment. *Bull Pan Amer Health Organiz 28(2): 144-152,*1994.

Weigel MM, Armijos RX. The traditional and conventional medical treatment of cutaneous leishmaniasis in rural Ecuador. *Pan American Journal of Public Health*, 10(6), pp395-404, 2001.

World Health Organization. *Control of the leishmanisis.* Geneva:WHO;1990. (Technological report series, 793).

Zachariadis O, Cassidy JP, Brady J, Mahon BP. γδ T Cells regulate the early inflammatory response to *Bordetella pertussis* infection in the murine respiratory tract. *Infection and Immunity*, 74(3):1837-1845, 2006.

GLOSSARY

Adjuvant. Is an agent that may stimulate the immune system and increase the response to a vaccine, without having any specific antigenic effect in itself.

Catechin. Is a polyphenolic antioxidant plant metabolite. The term catechin is also commonly used to refer to the related family of flavonoids and the subgroup flavan-3-ols (or simply flavanols).

Leishmaniasis. Is a disease caused by protozoan parasites that belong to the genus Leishmania and is transmitted by the bite of certain species of sand fly (subfamily Phlebotominae). Two genera transmit Leishmania to humans: Lutzomyia in the New World and Phlebotomus in the Old World.

Pathogenesis: The development of a disease. The origin of a disease and the chain of events leading to that disease.

Polyphenols. Are a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule.

FIGURES

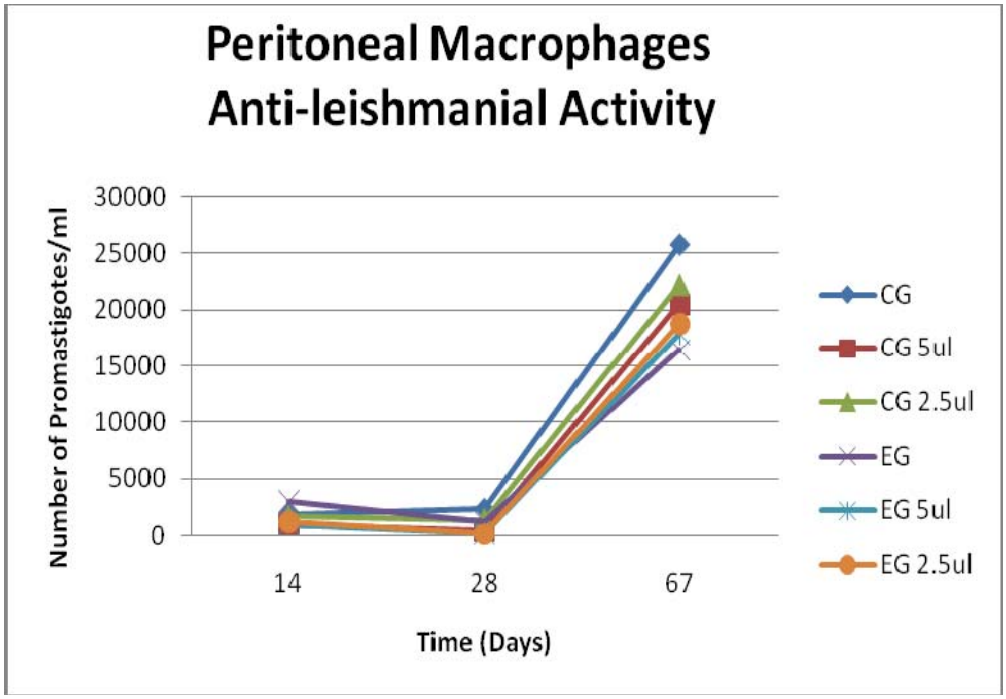

Figure 1. Peritoneal Macrophages Anti- leishmanial Activity between the EG and the CG.

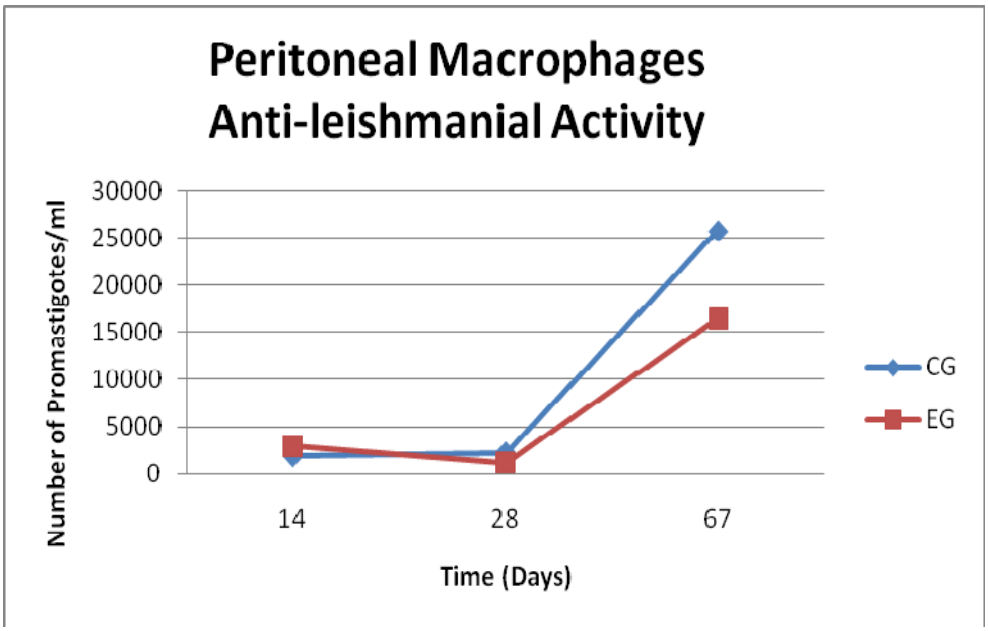

Figure 2. Peritoneal Macrophages Anti- leishmanial Activity between the EG and the CG with water.

Day 4 (t=0.495; p=0.645 NS)

Day 28(t=0.559; p=0.605 NS)

Day 67(t=1.279; p=0.269 NS)

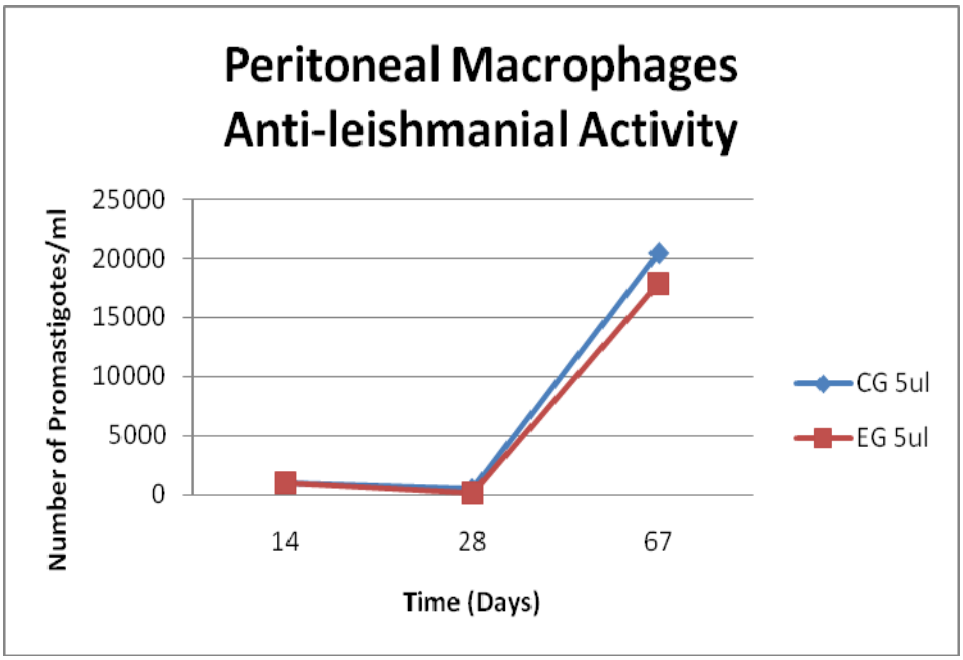

Figure 3. Peritoneal Macrophages Anti- leishmanial Activity between the EG and the CG with the experimental treatment of 5 microliters.

Day 4 (t=0.683; p=0.531 NS)

Day 28(t=-0.921; p=0.409 NS)

Day 67(t=-0.328; p=0.758 NS)

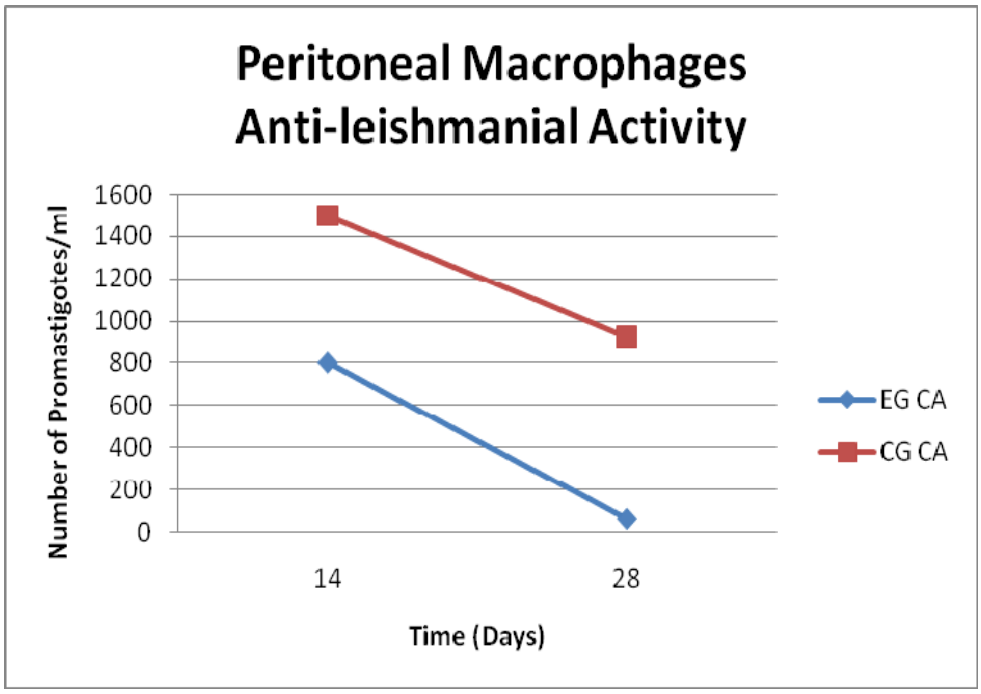

Figure 4. Peritoneal Macrophages Anti- leishmanial Activity between the EG and the CG with Caffeic Acid.

Day 4 (t=-0.871; p=0.432 NS)

Day 28(t=-1.520; p=0.202 NS)

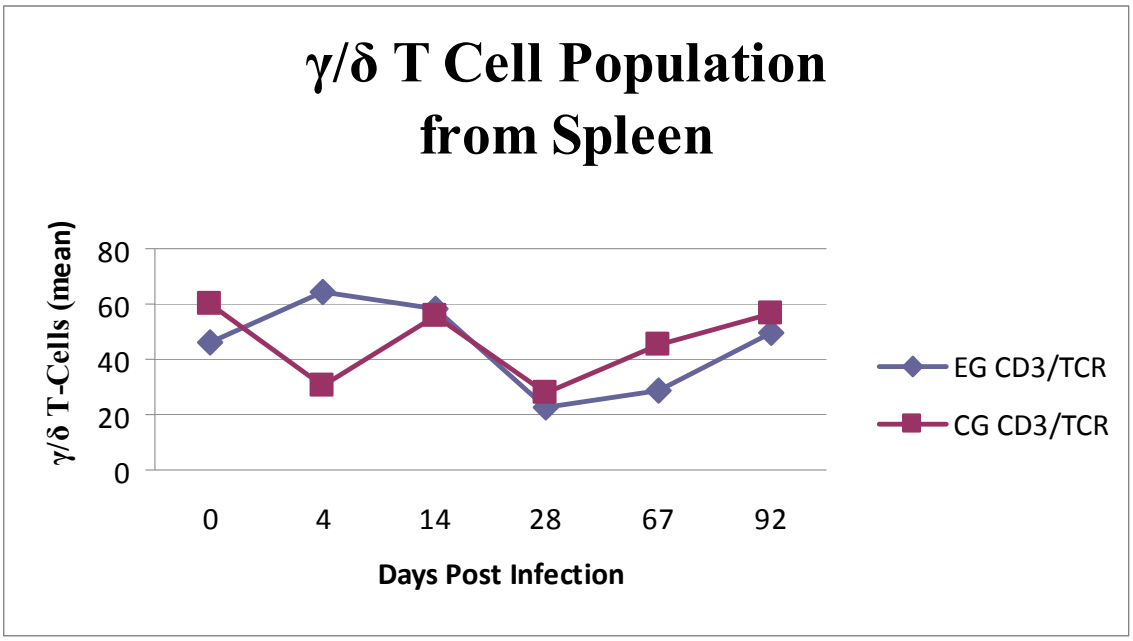

Figure 5. Flow Cytometric Analysis for CD3/TCR cells.

Baseline (NS)

Day 4 (NS)

Day 14 (NS)

Day 28 (NS)

Day 67 (NS)

Day 89 (NS)

Day 101 (NS)

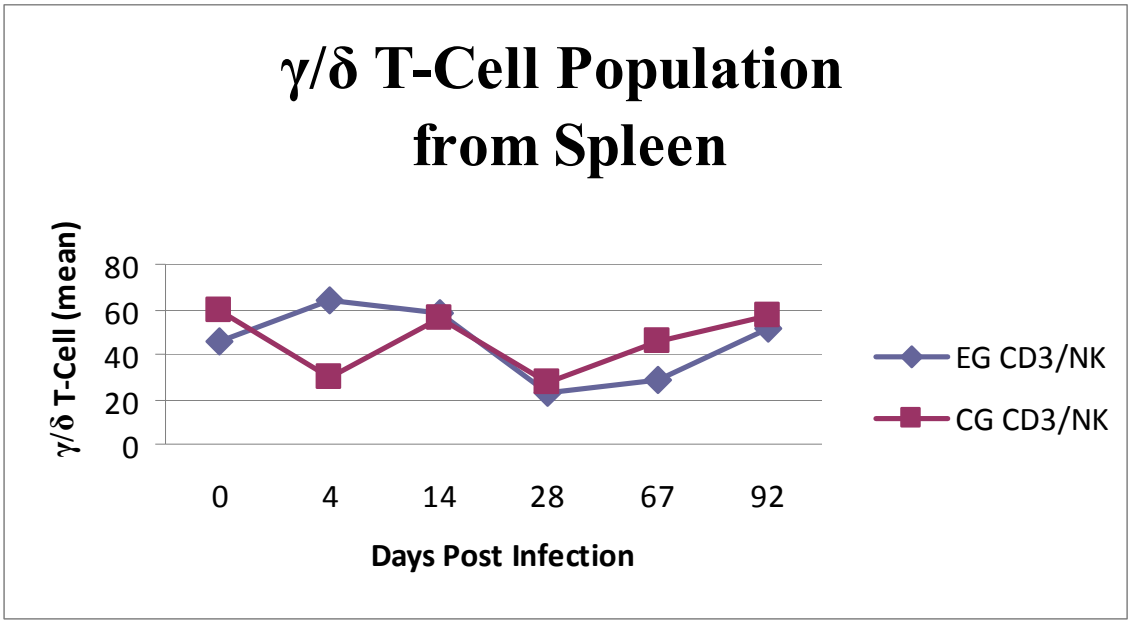

Figure 6. Flow Cytometric Analysis for CD3/NK Cells.

Baseline (NS)

Day 4 (NS)

Day 14 (NS)

Day 28 (NS)

Day 67 (NS)

Day 89 (NS)

Day 101 (NS)

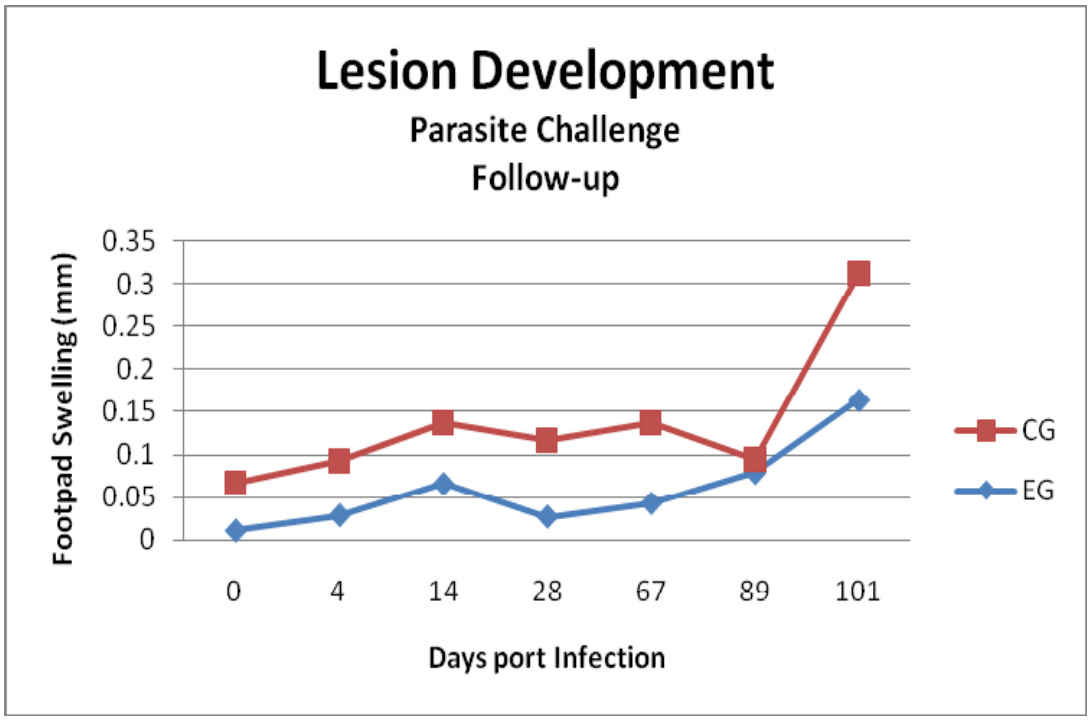

Figure 7. Lesion Development of parasitic challenge.

Baseline (NS)

Day 4 (NS)

Day 14 (NS)

Day 28 (t=-2.279; p=0.33)

Day 67 (t=-2.960; p=0.007)

Day 89 (NS)

Day 101 (NS)

CURRICULUM VITA

Alejandra Avila Blancarte was born in Ciudad Juarez, Chihuahua, Mexico. The first daughter of Gerardo Avila Avila and Hilda Isabel Blancarte Gonzalez and mother of Braulio Ariel Barragan Avila, she graduated from the Universidad Autonoma de Ciudad Juarez, Chihuahua in the fall of 2003 obtaining the degree of Bachelor's of Science in Human Nutrition and Food Sciences. Her undergraduate thesis title was Comparación de hábitos alimenticios actuales de estudiantes universitarios con el recordatorio de sus prácticas alimentarías infantiles. After completion of her bachelor's degree, Alejandra pursue the Master of Health Promotion at the University of Texas at El Paso.