172851

CELL MEDIATED IMMUNE RESPONSE IN COCKERELS UNDER TEMPERATURE STRESS

SAVITHA. M. K.

Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

2008

Departmert of Veterinary Physiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

DECLARATION

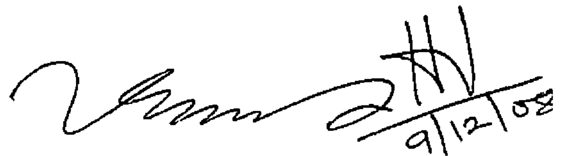
I hereby declare that the thesis entitled **"CELL MEDIATED IMMUNE RESPONSE IN COCKERELS UNDER TEMPERATURE STRESS"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy,
09.12.2008

SAVITHA M.K.

CERTIFICATE

Certified that this thesis, entitled **"CELL MEDIATED IMMUNE RESPONSE IN COCKERELS UNDER TEMPERATURE STRESS"** is a record of research work done independently by **Savitha M.K.**, under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.



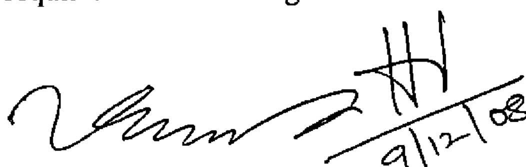
Dr. V. Ramnath,

(Chairman, Advisory Committee),
Associate Professor,
Department of Veterinary Physiology,
College of Veterinary and
Animal Sciences, Mannuthy.

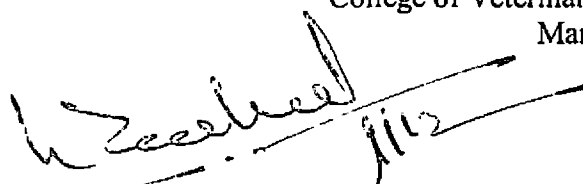
Mannuthy,

CERTIFICATE

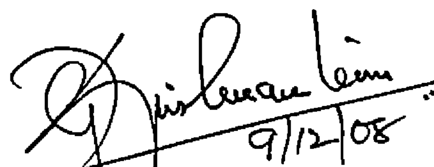
We, the undersigned members of the Advisory Committee of **Savitha M.K.**, a candidate for the degree of **Master of Veterinary Science in Physiology**, agree that the thesis entitled **"CELL MEDIATED IMMUNE RESPONSE IN COCKERELS UNDER TEMPERATURE STRESS"** may be submitted by **Savitha M.K.**, in partial fulfilment of the requirement for the degree.



Dr. V. Ramnath,
(Chairman, Advisory Committee)
Associate Professor,
Department of Veterinary Physiology,
College of Veterinary and Animal Sciences,
Mannuthy.



Dr. K.P. Sreekumar,
Professor and Head,
Department of Veterinary Physiology,
College of Veterinary and
Animal Sciences, Mannuthy.
(Member)



Dr. G. Krishnan Nair,
Professor and Head,
Department of Veterinary Microbiology,
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr. K.K. Jayavardhanan,

Associate Professor,
Department of Veterinary Biochemistry,
College of Veterinary and Animal sciences,
Mannuthy.
(Member)

11.2.09
External Examiner
(V. LEELA)

ACKNOWLEDGEMENTS

I express my heartfelt gratitude to the chairman of my advisory committee, Dr. V. Ramnath, Associate Professor, Department of Veterinary Physiology, for his strong guidance, constant support, keen interest and affectionate encouragement. I gratefully acknowledge the continuous encouragement and inspiration given by him throughout the period of this work.

I express my sincere gratitude to Dr. K.P. Sreekumar, Professor and Head, Department of Veterinary Physiology, a member of my advisory committee, for his valuable suggestions, expert advice, help and encouragement extended to me to carry out this study.

With immense pleasure, I depict my sincere thanks to Dr. G. Krishnan Nair, Professor and Head, Department of Veterinary Microbiology and Dr. K. K. Jayavardhanan, Associate Professor, Department of Veterinary Biochemistry for their constructive criticism, supporting attitude and guidance rendered to me as members of my advisory committee.

I am cordially obliged to Dr. P.R. Chandrasekhar, Professor and Head, Instrumentation Centre, Dr. P. Suresh Kumar, Professor and Head, Radiotracer Laboratory, Kerala Agricultural University and Dr. Sujatha. K. S, Associate Professor and Head, Department of Statistics for their affectionate encouragement, pleasant co-operation and valuable help.

I express my sincere thanks to Dr. K. Karthiayini, Associate Professor, Dr. V. Beena and Dr. Aziz Zarina, Assistant Professors, Department of Veterinary Physiology, for their personal attention, warm friendship and affectionate encouragement rendered during the period of study.

I greatly obliged for the facilities and financial help offered by Indian Council of Agricultural Research, as the study was a part of Ad-hoc ICAR project entitled "Immune responses of the chicken to environmental stress" implemented through the Department of Veterinary Physiology.

I am extremely thankful to Dr. E. Nanu, Dean, Faculty of Veterinary and Animal Sciences for providing me the facilities for my research.

The invaluable help rendered by Dr. S. Rekha, Senior Research fellow, Department of Veterinary Physiology, Mrs. Lini. C and Mr. Joshi. C. G. for their support, timely assistance and warm friendship.

I express my heartfelt thanks to all the non teaching staff of Department of Veterinary Physiology for all the help, comfort, warmth and cooperation given to me to complete this work.

I am in short of words to express my feeling of gratitude for the comfort, help and support of Drs. Mejo. K. R, Darsana. M. G, Princemon. K.S and Arul Mary Luveena during the various stages of my research work.

I cherish the spirit of understanding and personal encouragement rendered to me by my friends, Drs. Deepa Mary, Nimmy A. George, Bhadra. P. V, Seena. N. S and Archana S Nair.

I wish to extend my thanks to Drs. Rajeswary T, Jessy V, Anupama. K, Remya V, Indu K and Manjula V James for their whole hearted support and warm friendship throughout the course of this study.

No phrase or words can ever express my deep sense of love and gratitude to my beloved parents, brother, relatives and friends being always with me through thick and thin.

I treasure the invaluable friendship, deep sense of love and mental support given to me by my friends in the 'FLY' throughout the days of work.

Savitha M.K.

CONTENTS

Chapter	Title	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	3
3	MATERIALS AND METHODS	29
4	RESULTS	50
5	DISCUSSION	68
6	SUMMARY	81
	REFERENCES	84
	ABSTRACT	

LIST OF ILLUSTRATIONS

LIST OF TABLES

Table No.	Title	Page No.
1	Plasma corticosterone level, after 5 days of heat / cold stress (n=8)	52
2	Effect of heat / cold stress on peritoneal macrophage functions (n=8)	53
3	Effect of heat / cold stress on phytohemagglutinin (PHA) elicited cutaneous delayed hypersensitivity response (n=8)	55
4	Effect of heat / cold stress on leucocyte migration inhibition index (LMII) (n=8)	57
5	Effect of heat / cold stress on splenocytes proliferation assay (Mean ± SD values show decay of ^{3}H in cpm)	58
6	Effect of heat / cold stress on natural killer (NK) cell activity (Mean ± SD values show decay of ^{51}Cr in cpm)	59
7	Effect of heat /cold stress on per cent cell lysis of K562 cells through natural killer (NK) cell activity	61
8	Effect of heat / cold stress on antibody dependent cellular cytotoxicity (ADCC) response (Mean ± SD values show decay of ^{51}Cr in cpm)	63
9	Effect of heat / cold stress on antibody dependentcellular cytotoxicity (ADCC) mediated cell lysis of SRBCs	64
10	Effect of heat / cold stress on antibody dependent complement mediated cytotoxicity (ACC) (n=8)	66

LIST OF FIGURES

Figure No.	Title	Page No.
1	Effect of heat / cold stress on natural killer (NK) cell activity against K562 cells	62
2	Effect of heat / cold stress on antibody dependent cellular cytotoxicity (ADCC) against SRBCs	65
3	Effect of heat / cold stress on antibody dependent complement mediated cytotoxicity (ACC) against EAC cells	67

LIST OF PLATES

Plate No.	Title	Between Pages
1	Macrophages (Giemsa stained 100X) with engulfed SRBC/s Fig.(i) Macrophage with engulfed SRBC Fig.(ii) A macrophage with two engulfed SRBCs	53&54
2	Fig.(i) Leucocyte migration inhibition test A. represents antigen (BSA) free well with WBCs B. represent wells with both antigen and WBCs Fig.(ii) Antibody dependent complement mediated cytotoxicity (ACC) assay A. represent unstained live cells (40X) B. represent dead EAC cells stained with trypan blue (40X)	57&58

Introduction

1. INTRODUCTION

In tropical and arid areas, the variation in the environmental temperature is the major stress factor for animals and birds (Silanikove, 1992 and Mashaly *et al.*, 2004). Stress stimulates the hypothalamo-hypophyseal-adrenal axis and increases corticosteroid secretion which alters susceptibility of animals including chickens to infectious diseases, resulting in production loss at temperatures above or below the thermo-neutral zone (Brown and Nedtor, 1973). Since, the range of the comfort zone or thermo-neutral zone of birds is narrow, they are highly susceptible to heat and cold environmental stress (Siegel, 1995). The present breeds of chicken which have undergone intensive selection for higher production might have lost their ability to cope with stress (Mashaly *et al.*, 2004).

During the stress period, dietary nutrients are shunted away from growth, while body nutrients are broken down and amino acids are used by specific cells to synthesize critical proteins such as acute phase proteins and antibodies. They not only act against stress causing agents, but also improve defense mechanisms. Vitamin C plays a pivotal role in decreasing the continued synthesis of corticosteroids from the adrenal cortex. Although poultry can synthesize vitamin C, synthesis becomes inadequate under stressful conditions (Sykes, 1978 and McDowell, 1989).

Several methods are available to alleviate the negative effects of high environmental temperature on performance of poultry. The high cost and impracticability of cooling animal sheds, lead to stress alleviation through dietary manipulations (Ramnath *et al.*, 2007). Through dietary supplementation of α-tocopherol and mannitol (Niu *et al.*, 2007) and by perilla seeds, successful management of heat stress had been reported in rats (Lee *et al.*, 2007). Preparations like Livol and Zeestress possess hepatoprotective property (Parida *et al.*, 1995) that they also reduce stress in intensively housed chickens (Wheeler, 1994), as well as in layers during summer (Roy *et al.*, 1996).

Adaptive immunity can be categorized as cell mediated immunity (CMI) and antibody mediated immunity (Davison and Lickiss, 1979). Cell mediated immune responses are handled within the body by natural killer (NK) cells, cytotoxic T-lymphocytes and macrophages. They have got a key role not only in tumour cell destruction but also in the elimination of antigens through interactions with antibody, complement and cytokines (Manu and Kuttan, 2007).

One of the main strategies of using herbal products is to increase the body's natural resistance to pathogens rather than directly neutralizing the agents themselves. Rasayanas are a group of non-toxic preparations commonly used in indigenous medical practice in India to improve health and longevity (Hanumanthachar and Milind, 2006 and Mohandas Rao *et al.*, 2006). Brahma Rasayana (BR) and Chyavanaprasha are two non toxic poly herbal preparations, made from the extracts of plants, claimed to be potent immunomodulators (Joseph *et al.*, 1999).

A lacuna of information exists with respect to the immunosuppressive effects of excess endogenous corticosteroids produced during heat / cold stress and the various immunomodulators that can be used to overcome the same. The present study is undertaken to determine the cell mediated immune responses of chickens subjected to temperature stress (high temperature-high humidity as well as low temperature- low humidity conditions), and the effects of BR supplementation as a cheap, cost effective immunomodulator in chicken during adverse environmental conditions.

Review of Literature

2. REVIEW OF LITERATURE

Both high and low environmental temperatures stimulate the hypothalamo-hypophyseal-adrenocortical axis, which may alter susceptibility of animals including the chicken to infectious diseases resulting in production loss. Herbal drugs are considered either non-toxic or less toxic than their synthetic counterparts. This has given impetus to screen herbs for their anti-stress ability. The traditional Indian system of medicine, Ayurveda, extensively uses plant derived compound formulations for the treatment of various ailments.

Several marketed herbal preparation such as Brahma Rasayana, Chyavanaprasa, Triphala and Septilin have antifungal, antibacterial, antidiabetic, antipyretic, antioxidant, anticlastogenic and hepatoprotective properties.

2.1 BODY TEMPERATURE AND BODY WEIGHT AS INFLUENCED BY TEMPERATURE STRESS

Pardue *et al.* (1985) conducted a study to determine the role of ascorbic acid (AA) in male chickens exposed to high temperature (38^0C). They found that heating increased mortality, where as AA (1000 ppm) supplementation improved livability. The weight loss of AA treated birds was only 0.67 per cent of their initial weight, whereas that in non- treated birds it was 3.8 per cent.

Dabbert *et al.* (1997) studied the effects of acute thermal stress on the immune system of the northern bobwhite (*Colinus virginianus*). They noted that the heat stress (cycled from 30.8 to 39.0^0C over 24 h) had no significant effect on body mass, whereas, cold stress (cycled from 3.6 to 20^0C over 24 h) resulted in loss of an average 9.1 ± 0.9 g of body mass and thermoneutral birds (constant 21^0C) gained an average of 2.4 ± 1.6 over the four days trial.

McKee *et al.* (1997) studied the effect of supplemental AA on the energy conversion of broiler chicks during heat stress (33.40 ± 1^0C) and they noticed that

heat exposure depressed weight gain, while AA supplementation increased weight gain. They also suggested that supplemental AA influenced body energy stores that are used for energy purposes during periods of stress.

Yahav *et al.* (1997) examined the broiler chicks exposed to 36 ± 1^0C; 70-80 per cent relative humidity (RH) for 24 h at the age of 5 days and noticed decreased weight gain. At the age of 42 days, chickens challenged with acute heat stress of 35.0 ± 1^0C; 25-30 per cent RH resulted in hyperthermia (44.7 ± 0.4^0C) and increased mortality.

Puvadolprid and Thaxton (2000) developed a model to study stress in chickens. They found that continuous delivery of ACTH at 8.00 IU/kg bw/day for 7 days caused significant decrease in body weight in chicken.

Mehmet *et al.* (2005) studied the effects of AA on the performance of Japanese quails reared under hot conditions. They reported that oral administration of AA (500.00 mg/kg) had significant gain on body weight and feed consumption until fourth week.

2.2 PHYSIOLOGICAL VALUES AS INFLUENCED BY TEMPERATURE STRESS

Regnier and Kelley (1981) studied the influence of hot and cold ambient temperature on peripheral lymphocyte responses of chicken and found that induced wattle swelling were reduced in birds exposed to high temperature when compared to those birds maintained at low temperature.

Pardue *et al.* (1985) studied the role of AA in male chickens exposed to high temperature (38^0C). They noticed that heating increased plasma corticosteroids and AA treated heat stressed chicks showed mean corticosteroid values of 8.8 ng/ml compared to 18.1 ng/ml in untreated chicks.

Hayashi and Kikuchi (1989) studied the time relationship between ambient temperature change and antigen stimulation on immune responses of mice. They observed an elevation of the blood corticosterone levels for only the first 3 days after a temperature shift from 25^0C to 8^0C, but the elevation was persisted for 10 days after a temperature shift from 25^0C to 36.5^0C, suggesting that the blood corticosterone level contributes to the duration of the effects of temperature shifts to immune responses in mice.

Widerman *et al.* (1994) concluded that heat acclimated broilers exposed to high environmental temperature were more resistant to heat stress and they consumed more water during heat stress than non-acclimated controls.

Yahav *et al.* (1997) studied the haemodynamic changes in broiler chickens during exposure to constant temperatures (10 to 35^0C). They found that PCV was significantly high at low constant temperatures (10^0 and 15^0C), whereas at high temperatures (30^0 and 35^0C) plasma volume (PV) was significantly high. A linear relationship between hematocrit and heart weight was observed and indicated an adaptation of heart mass to changes in cardiac output and hematocrit to meet the demands of increased basal metabolic rate. They postulated that changes in PCV are probably related to modulation of the supply of oxygen to accommodate changes in heat production and the significant hypervolemia observed at high temperatures occurred to provide the fluid needed for heat dissipation by panting.

Altan *et al.* (2000) studied the effect of heat stress on some blood parameters in broilers. They found that exposure of broilers to 39.0^0C, significantly increased rectal temperature, heterophil and basophil proportions, and the H/L ratio increased from 0.25 to 0.43, where as, proportion of monocyte and lymphocyte decreased. It was demonstrated that acute heat stress did not affect the haematocrit values or eosinophil proportion.

Yadav *et al.* (2000) examined the lack of response of laying hens to relative humidity at high ambient temperature. The laying hens of different ages were exposed to 25^0C for 3 weeks and thereafter 35^0C for 1 week at different relative humidities (40 -70 per cent). They noted a decline in food intake, while an increase in water consumption with increasing ambient temperature, whereas egg production was unaffected with reduction in various egg qualities. Thus they concluded that the ambient temperature was the main environmental factor affecting young and older layers.

Oladele *et al.* (2001) determined the effect of season and sex on the PCV, haemoglobin (Hb) and total proteins in pigeons and they noted, the highest PCV and Hb during rainy season while, lowest was seen in hot season and they found these values were higher in males than in females. They also noticed higher total protein during hot and rainy season.

Shinder *et al.* (2002) examined the influence of short repetitive cold exposure at an early age (15^0C for 3 h at 3 and 4 days of age) and at 21 days of age on chicken's thermotolerance. They noted a significant elevation in plasma corticosterone concentration and upon chronic exposure it was remained at a significantly lower level.

Djordjevic *et al.* (2003) studied the activation of adrenocorticotropic hormone (ACTH) and corticosterone release in response to various stressors in rats. They noticed a significantly increased ACTH plasma levels in rats exposed to different stressors. Exposure to heat (38^0C, for both 20 and 60 min), produced the largest increment in plasma ACTH levels. They also noticed that fasting and cold stressors were less potent in elevating plasma ACTH concentration. They concluded that exposure to ambient temperature of 38^0C appears to be the strongest stressor activating the hypothalamo-hypophyseal-adrenal axis.

Graczyk *et al.* (2003) found that in chickens injected with triple dose of ACTH (0.01 mg/100 g bw), there was a decreased proportion of lymphocytes and increased

heterophils. They concluded that increased level of corticoids in the serum led to the increased values of H/L index.

Hangalapura *et al.* (2004) studied the effect of cold stress (CS) on immune responses and plasma adrenal hormone levels in chickens. A significant enhancing effect of CS was found on lymphocyte proliferation (LP). A significant dose-dependent suppressive effect of CS was found on plasma corticosterone levels. They suggested that CS did not affect specific antibody responses, but may have a modulating effect on cellular immunity and plasma corticosterone levels, depending on the duration of the stress. They concluded that there was an inverse relationship between LP and corticosterone level.

Mashaly *et al.* (2004) evaluated the effect of heat stress on production parameters and immune responses of commercial laying hens. They reported a significant reduction in body weight, feed consumption, egg production, egg weight and shell thickness in the heat stressed group. They also observed a significant inhibition in total white blood cells, and antibody production whereas, mortality was higher in the heat stressed group compared to control groups. These results indicated that heat stress not only adversely affected production performance but also inhibited immune function.

Mehmet *et al.* (2005) studied the effects of AA on some blood parameters of Japanese quails reared under hot conditions. They reported that oral administration of AA (500 mg/kg) did not affect the blood pH, pCO_2, PCV or Hb in quails.

Abbas *et al.* (2007) examined the effect of lighting and melatonin addition (40 ppm) to the diet in alleviating negative impact of heat stress on the immune response in broiler chickens. Chickens exposed to continuous heat stress (35^0C for 4 wks) had significantly higher levels of plasma corticosterone than the chickens under control temperatures.

2.3 ROLE OF MACROPHAGES IN CELL MEDIATED IMMUNE RESPONSE

Walker and Demus (1975) evaluated antibody dependent cytolysis of chicken erythrocytes by an *in vitro* established mouse peritoneal macrophage cell line. They reported that the macrophages phagocitized and lysed ^{51}Cr labeled chicken erythrocytes in the absence of complement but they proved that the phagocytic process was functionally distinct from the cytolysis and it was demonstrated by enhanced cytolysis in the presense of iodoacetate, an inhibitor of phagocytosis.

Blanckmeister and Susdorf (1985) studied macrophage activation by cross-linked dextran. Cross- linked dextran (Sephadex) is an effective inducer yielding large amount of macrophages after peritoneal injection into mice and also activates its functions. They compared the results of dextran administration, saline, thioglycollate and complete freund's adjuvant, and observed that the glass adherence and migration in agarose were significantly increased in dextran administered group. Thus it was concluded that cross-linked dextran in the form of Sephadex G-25 not only functioned as peritoneal exudate inducer but also as an effective macrophage activator.

Saxena *et al.* (1991) reported macrophage migration as an index of immune status. The peritoneal macrophages from mice and guinea pigs pretreated with freunds complete adjuvant (FCA) when packed in a glass capillary and placed in a migration chamber migrated to a larger area than macrophages from normal untreated animals. The ratio between the areas of migration of macrophages from FCA treated animals and of macrophages from untreated animals was above 3.0 under optimum conditions. They also noted a close correlation between macrophage migration and delayed hypersensitivity response in animals sensitized with ovalbumin or sheep red blood cells and an increased phagocytic activity of macrophages of immunostimulant-treated animals.

Miller and Qureshi (1992a) evaluated the induction of heat-shock proteins and phagocytic function of chicken macrophage following *in vitro* heat exposure. They found out that the macrophages exposed to 45^{0}C for 30 min and 60 min were significantly depressed in phagocytosis of uncoated sheep erythrocytes (SE) under 45^{0}C incubation. However, phagocytosis of antibody-coated SE was not affected when compared to 41^{0}C control cultures. This study suggested that in chicken peritoneal macrophages the Fc-mediated phagocytic function significantly depressed their nonspecific phagocytosis, while higher number of Fc receptors noticed on cell surface maintained phagocytic function against antibody coated SE.

Neldon- Ortiz and Qureshi (1992) evaluated the effects of direct and microsomal activated aflatoxin B1 on chicken peritoneal macrophages *in vitro*. They studied the effects of aflatoxin B1 (AFB1) at different concentrations directly and that of AFB1 in the presence of chicken microsomal mixed function oxidase system (MFO) at different concentrations. They found out that the direct *in vitro* exposure to AFB1 caused no detrimental effects on per cent phagocytic cells or the number of internalized sheep red blood cells (SRBCs) however, significant reduction in phagocytosis and internalization of SRBCs when MFOs were added to cultures in lower doses of AFB1 suggesting that microsomal activated AFB1 caused significant alterations in macrophage phagocytic properties.

Courreges *et al.* (1994) studied the *in vitro* antiphagocytic effect of *Melia azedarach* leaf extracts on mouse peritoneal exudate cells and observed an inhibition of phagocytosis of opsonized sheep erythrocytes which was both dose and time dependent as it was reverted 48 h after removing the extract from the culture medium.

Dabbert *et al.* (1997) noticed that the total peripheral blood leucocyte counts of northern bobwhite (*Colinus virginianus*) subjected to cold stress (cycled from 3.6 to 20.0 ^{0}C over 24 h) for four consecutive days were 30 percent lower than those in the thermoneutral condition. Cold stress brought an increase in the relative percentage of monocytes but decreased the relative percentage of heterophils in peripheral blood

smears. They concluded that cold stress might have increased the activity of phagocytic leucocytes, which are important in resistance of birds to bacterial pathogens.

Gore and Qureshi (1997) reported the enhancement of humoral and cellular immunity by vitamin E after embryonic exposure. They injected different levels of vitamin E (VE) in turkey and broiler embryos into the amnion and their effects on humoral and cellular immunity were compared with controls. The poults received 10 IU VE had higher numbers of Sephadex elicited inflammatory exudated cells, greater percentage of phagocytic macrophages and numbers of sheep red blood cells per phagocytic macrophage than in control poults at 4 wk of age.

Peterson *et al.* (1999) found out that the *in vitro* exposure with β-hydroxy- β-methylbutyrate (HMB) enhances chicken macrophage growth and function. They noted that HMB in various concentrations stimulated the proliferation of macrophages. They also noticed that 20 μg HMB increased nitrite production by 44.1 per cent over the controls and the phagocytic potential of macrophages exposed to 40 μg and 80 μg HMB exhibited 31.7 per cent and 14.4 per cent higher phagocytosis respectively over controls.

Hangalapura *et al.* (2004) examined the effects of cold stress on overall immunity of chicken lines divergently selected for antibody responses. They studied the *in vitro* lymphocyte proliferation response to concanavalin A (Con A) and keyhole limpet hemocyanin (KLH) as measures of cell-mediated immunity, production of zymosan- induced reactive oxygen intermediates as a measure of phagocytosis, and body weight gain as a measure of a production trait in 3 lines of chickens subjected to cold stress (CS) of 10^0C for 0, 2, or 7 day. They found that the innate part of the immune system (phagocytes) responded immediately to CS irrespective of the genetic background as the productions of reactive oxygen intermediates were inconsistent. Day 7 of CS significantly enhanced cellular

immunity to Con A *in vitro* indicating that prolonged CS affected the cellular adaptive immune response.

2.4 CUTANEOUS DELAYED HYPERSENSITIVITY RESPONSE

Regnier and Kelley (1981) revealed that heat and cold stress suppress *in vivo* and *in vitro* cellular immune responses of chickens. They studied the influence of hot and cold ambient temperatures on cellular immune responses of chickens utilizing dinitrofluorobenzene- and PHA induced wattle swelling. They found that much of the reduced wattle swelling in birds exposed to 36^0C for 5 days when compared to that in chickens maintained at 26^0C. The PHA-induced lymphocyte transformation was also suppressed in chickens exposed to either heat or cold stress for 5 days. It was concluded that chronic heat and cold stress impaired expression of contact sensitivity *in vivo* and proliferation of T lymphocytes *in vitro* in the avian species.

Brake *et al.* (1988) studied the effect of cortisol on cutaneous basophil hypersensitivity to phytohemagglutinin (PHA-P) in chickens. Four injections of 2 mg cortisol in corn oil/500 g bw i.m. were given, and 12 h after the last injection, PHA-P 100 µg/ 50 µl saline was administered intra dermally in the right wattle and 50 µl of sterile saline in the left wattle as control. They reported that the peak responses occurred within 18-24 h after PHA-P challenge (5.87 mm and 5.47 mm). The results indicated that cortisol enhanced the cells involved in cutaneous basophil hypersensitivity responses. This was in contrast to reported immunosuppressive effects of other glucocorticoids.

Corrier and DeLoach (1990) evaluated the cell mediated cutaneous basophil hypersensitivity in young chickens by an interdigital skin test. They observed that the cutaneous basophil hypersensitivity response elicited by PHA-P intradermally in chicken were maximum after 12 h post injection, which remained constant at 24 h post injection. The post injection thicknesses ranged from 1.43 to 1.83 mm in 100 µg

PHA-P injection in various age groups whereas, 1.28, to 1.78 mm in 200 µg PHA-P injected birds.

Peterson *et al.* (1999) reported the enhancement of cellular and humoral immunity in young broilers by the dietary supplementation of HMB. They found out that the cutaneous basophilic hypersensitivity in broilers receiving HMB treated diets were lower than the response by broilers receiving control diet, 24, 48, and 72 h post mitogen injection. They also reported that the phagocytic potential of the macrophage was not affected by HMB supplementation. However, nitrite production by the macrophages was elevated over the controls when HMB was supplemented in the diet. The results suggested that the phagocytic ability and respiratory burst activity of polymorphonuclear leukocytes receiving HMB was elevated over the controls.

Fulzele *et al.* (2003) studied the immunomodulatory activity of *Haridradi ghrita* (HG) in rats. They tested the cellular immune response by footpad swelling and neutrophil adhesion tests in rats, orally administered with HG at different doses. They observed that the oral administration of HG showed an increased neutrophil adhesion and delayed hypersensitivity (DTH) responses to sheep RBCs. The DTH response was 14.50 per cent increase in paw volume in comparison to the untreated control group having 6.01 per cent.

Abbas *et al.* (2007) examined the effect of lighting and melatonin addition (40 ppm) to the diet in alleviating negative impact of heat stress on the immune response in broiler chickens. They found that the cutaneous basophil hypersensitivity response as measured by increase in wattle thickness, 24h following PHA-P injection was significantly lower in CHS birds and those receiving melatonin.

2.5 LEUKOCYTE MIGRATORY RESPONSE

Belsheim (1981) reported the technical aspects of a modified leukocyte migration under agarose technique (LMAT). He/She noted that temperature, pH, gas

mixtures, time allowed for migration and storage of leukocyte suspensions, presence of divalent cations, and proteins in the leukocyte suspension were the critical factors for the migration response. The serum behaved satisfactorily as a chemotactic factor only in freshly–made agar matrices indicating the development of anticomplementory activity in agar.

Chhabra and Goel (1981) examined the immunological response of chickens to *Mycoplasma gallisepticum* infection. The cellular immune response as indicated by significant leukocyte migration inhibition (LMI) was demonstrated as early as one week postinfection (PI). But the maximum LMI of 36.4 per cent was observed at seven weeks PI.

Gilliland *et al.* (1982) used a direct agarose LMI assay for studying DTH in avian species. The antigen specific LMI was demonstrated in chickens with DTH to purified protein derivative of Mycobacterium (PPD) and ferritin. They also found a good relation between LMI and DTH, measured by the delayed wattle reaction. The bacterial lipopolysaccharide (LPS) (in µg) quantities were found to inhibit *in vitro* migration of chicken leukocytes.

Pawan *et al.* (1991) evaluated the cell-mediated immune response of chickens vaccinated with Newcastle disease virus by the under- agarose leukocyte- migration-inhibition technique. They obtained a significant cell-mediated immune (CMI) response from chickens vaccinated with Newcastle disease vaccine. They reported that the presence of the CMI response always correlated with the presence of serologic immune response and vice versa but the quantitative level of the CMI response was not always corresponded to the quantitative level of the serologic immune response. They demonstrated that under agarose LMI test was a reproducible and relatively easy assay performed to evaluate CMI response.

2.6 BLASTOGENIC RESPONSE OF SPLENOCYTES TO MITOGENS

Sharma and Lee (1983) studied the effect of infectious bursal disease on natural killer cell activity and mitogenic response of chicken lymphoid cells. While, NK cell activity was not much influenced, the blastogenic response of spleen cells to PHA was depressed during the first 2 wks of infection. They observed several suppressor cells involvement in mitogenic hyporesponse similar to macrophages and the removal of these cells restored the mitogenic reponse. They concluded that the reduced mitogenic response of lymphocytes in IBDV- infected chickens was not due to a lack of functional-T cells but was due to macrophage-like suppressor cells get activated during early stages of IBDV infection.

Chi *et al.* (1984) studied the effect of temperature and lymphokines on mixed lymphocyte and mitogen responses of chicken lymphoid cells *in vitro* and reported that the responses at 40^0C were much higher than at 37^0C. They concluded that it was due to the faster kinetics of the response at 40^0C level.

Cochran and Baxter (1984) conducted a study on macrophage-mediated suppression of T lymphocyte proliferation induced by oral carrageenan (CGN) administration. They administered CGN (a potent inhibitor of macrophage mediated cell response) orally at 5mg/kg or 50 mg/kg dose to rats and reported that the low dose suppressed spleenic T cell proliferation than the other dose to PHA or Con A. Restoration of the PHA mitogenic response after the removal of adherent cells suggested that the presence of a macrophage-mediated mechanism suppressed lymphocyte activation. Supernatants from peritoneal exudates macrophages and resident macrophages obtained from CGN - fed rats, also suppressed PHA- induced spleen cell mitogenesis. These data indicated that low doses of orally administered CGN stimulated a population of macrophages to actively suppress T lymphocyte proliferation.

Kline and Sanders (1984) studied the suppression of Con A mitogen- induced proliferation of normal spleen cells by macrophages from chickens with hereditary muscular dystrophy. The spleen cells from chickens with hereditary muscular dystrophy (MD) yielded low blastogenic response to the mitogen Con A and exhibited normal mitogen stimulated blastogenic response to PHA. The addition of MD spleen cells to normal spleen cells caused a marked suppression of the Con A response of the normal cells while unaffected the PHA response. They concluded that the suppressive activity requires viable MD cells and is contact mediated.

Letwin and Quimby (1986) studied the effects of Con A, PHA, PWM and LPS on the replication and immunoglobulin synthesis by canine peripheral blood lymphocytes (cPBL). They reported that cPBLs secreted more IgG than IgM in response to mitogens and was measured with ^{3}H thymidine uptake. At 290 ng/ml ConA dose where there was increased ^{3}H thymidine incorporation with a decrease in IgG secretion; it appeared that an active suppression of the IgG response was induced by ConA.

Niwano *et al.* (1990) demonstrated a suppressed peripheral blood lymphocyte blastogenesis in pre-and postpartal sheep by chronic heat-stress, and suppressive property of heat-stressed sheep serum on lymphocytes. They observed that the mitogen induced lymphocyte response was reduced both before and after parturition in heat stressed sheep when compared to the control animals under thermoneutral conditions. They also reported the suppression of lymphoblastogenesis (in man, sheep, bovine) when the serum from heat-stressed sheep was added to it. The heat-stressed sheep serum did not inhibit IL-2 production by PHA-stimulated human peripheral blood lymphocytes. These results indicated that the immunosuppression of heat-stressed sheep was in part mediated by some serum factors other than complements and heat labile proteins that can modulate T-cell function in a species nonspecific manner.

Lowenthal *et al.* (1994) examined the development of T cell mediated immune response in the chicken. The spleenic T cells from chickens of various ages were cultured *in vitro* and stimulated with various mitogens like Con A and PHA. They observed that the T cells obtained from adult chickens proliferated extensively while the T cells from day old chickens failed to proliferate. The reactivity to mitogens gradually developed between days 2 and 4, and by 1 week of age it was equivalent to that observed with T cells from adult hens. Even though the T cells from day old chicks were functionally immature they were found to be phenotypically mature and capable of binding mitogens as T cells from adult birds. This might be due to the production of a soluble inhibitor which prevented the proliferation of stimulated adult T lymphocytes in chicks.

Trout andMashaly (1995) examined the effects of *in vitro* corticosterone on chicken T- and B- lymphocyte proliferation. They studied the effects of corticosterone addition to cultures, on Con A and PWM - induced lymphocyte proliferation of peripheral blood lymphocytes. Corticosterone, when added to the lymphocyte cultures either 2 h before or 2 h after the addition of the mitogen, suppressed cell proliferation. The results also indicated that after early activation events, initiated by mitogens, lymphocytes were less sensitive to the effects of corticosterone. There existed different sensitivities to corticosterone in the cell populations that responded to these mitogens.

Shephard and Shek (1998) studied the influence of cold exposure on immune function of small mammals. The data suggested that the acute effect of chilling suppressed several cellular immune responses like lymphocyte proliferation, natural killer (NK) cell count, cytolytic activity. However, adaptation to the cold stimulus develops over the course of 2-3 weeks.

Sijben *et al.* (2000) studied the immunomodulatory effects of indomethacin and prostaglandin E2 on primary and secondary antibody responses in growing layer

hens. The effects of prostaglandinE2 (PGE2) and indomethacin, an inhibitor of PGE2 oxygenase were studied in 35 and 70 d old layer hens. In mitogen- induced T-cell proliferation *in vitro* assay, PGE2 increased PHA-induced lymphocyte proliferation whereas indomethacin decreased Con A and PHA-induced lymphocyte proliferation. They explained the decrease in lymphocyte ^{3}H uptake by increasing concentration of indomethacin in terms of its toxic properties at higher concentrations.

Puthpongsisriporn *et al.* (2001) evaluated the effects of vitamin E and C supplementation on egg production, *in vitro* lymphocyte proliferation, and antioxidant status of laying hens during heat stress (21^{0}C to 35^{0}C) for 3 wks. They found that the lymphocyte proliferative responses to Con A and *Salmonella typhimurium* lipopolysaccharide (LPS) were greater in hens fed with 45 and 65 IU of vitamin E/kg during heat stress. They also found that supplementation of 65 IU of vitamin E/kg or 1,000 ppm vitamin C significantly enhanced *in vitro* lymphocyte proliferation to Con A and LPS, while the combination of these two brought about the highest *in vitro* responses indicating both vitamin E and vitamin C enhances immune responses during heat stress.

Koenen *et al.* (2002) studied the immunological differences between layer- and broiler- type chicken. They measured the *in vitro* cellular immune response by an antigen- specific lymphocyte proliferation assay. The trinitrophenyl-specific cellular immune response was detectable in layer-type chicken, but not in broilers. Both types generated a non-specific cellular immune response, although in broilers it was lower than in layer- type chickens. The results suggested that the broilers are specialized in the production of strong short- term humoral response and layer-type chickens in a long- term humoral response in combination with a strong cellular response, which was in conformity with their life expectancy.

Sairam *et al.* (2002) determined the antioxidant and immunomodulatory properties of *E.officinalis* with the emphasis on lymphocyte proliferation, using chromium (Cr) as an immunosuppressive agent and they found Cr treatment resulted in enhanced cytotoxcity and free radical production. The result revealed that gooseberry inhibited apoptosis and DNA fragmentation induced by Cr and relieved the immunosuppressive effects on lymphocyte proliferation.

Rekha *et al.* (2003) investigated the role of Brahma Rasayana (BR) on the immune status in irradiated mice. They observed that ^{3}H- thymidine uptake by splenocytes from normal animals administered with BR orally (50mg/dose/animal for 10 days) was two times increased (717 ± 155 counts per minute (cpm)) than those from untreated normal mice (352 ± 55.6 cpm). The ^{3}H- thymidine uptake was increased in both groups (1627 ± 197 and 965 ± 249 respectively) when the cells were stimulated with PHA (6µg/ml). There was a reduction in ^{3}H - thymidine uptake by cells of irradiated mice in both treated and non-treated groups.

Thejass and Kuttan (2006) reported the augmentation of natural killer cell and antibody-dependent cellular cytotoxicity in Balb/c mice by sulforaphane, a naturally occurring isothiocyanate from broccoli through enhanced production of cytokines IL-2 and IFN-γ. They noticed that sulforaphane significantly increased the proliferation of splenocytes, and thymocytes by stimulating the mitogenic potential of mitogens such as Con A and PHA.

Abbas *et al.* (2007) examined the effect of lighting and melatonin addition (40 ppm) to the diet in alleviating negative impact of heat stress on the immune response in broiler chickens. They observed that the exposure of birds to chronic heat stress (CHS) for 35^{0}C for 4 wks can inhibit *in vitro* T cell proliferation with Con A, compared to the control group (24^{0}C). Melatonin in the diet increased the proliferation of T-cells in chickens raised in control temperatures but not resulted in a significant increase in CHS chicken where T-cell proliferation was inhibited.

Guruvayoorappan and Kuttan (2007) studied the effect of *Biophytum sensitivum* on the cell mediated immune response in mice. They found that the *B. sensitivum* (1, 5, 10 μg/ml) enhanced the proliferation of splenocytes by stimulating the mitogenic potential of both Con A (10 μg/ml) and PHA (2.5 μg/ml). The maximum response was observed with 10μg/ml as 2580 ± 269 cpm compared with mitogen free (1713±196 cpm). Con A produced a higher proliferation (4353 ± 650 cpm) when compared to control added with the preparation alone. The mitogenicity of PHA was also enhanced (4040 ± 126 cpm) compared to *B. sensitivum* control (2730 ± 265 cpm).

Sheeja and Kuttan (2007) reported that the modulation of natural killer cell activity, antibody-dependent cellular cytotoxicity, and antibody-dependent complement-mediated cytotoxicity by andrographolide in normal and Ehrlich ascites carcinoma- bearing mice. They found out that the admimistration of *Andrographis paniculata* extract (APE) and its isolated compound andrographolide (ANDLE) enhanced the mitogen- induced proliferation of splenocyte, thymocyte and bone marrow cells in normal as well as tumor-bearing animals.

2.7 ROLE OF LYMPHOCYTES IN CELL MEDIATED IMMUNE RESPONSE

Radwan *et al.* (1975) studied ^{51}Cr release microassay for measuring the cell mediated immune responses in chickens. They used $2x10^7$ lymphoid cells per ml of RPMI-1640 medium as effector cells and sodium chromate labeled chicken red blood cells (ChRBC) $2x10^5$ cells/ml RPMI-1640 as taget cells, making the spleen to- target cell ratio 100:1. Such samples were incubated at 37^0C and 41^0C for 20 h in 100μg of PPD/ml. They observed that with sensitized spleen cells, target cell lysis was increased by incubation at 41^0C as compared to 37^0C.

Koren and Kim (1978) studied the natural killing and ADCC are independent immune functions in the minnesota miniature swine against human myeloid and lymphoid tumor target cells. The adult specific pathogen- free and germfree animals exhibited normal levels of both NK cell and ADCC. They also examined the NK and

ADCC activities of peripheral blood lymphocytes from colostrums deprived newborn piglets and showed to be virgin immunologically. They also found that the newborn piglets exhibited normal ADCC but lacked NK activity. The differences in the ontogeny of the two activities suggested that they are distinct.

Yanagihara and Adler (1982) studied the inhibition of mouse natural killer activity by cyclosporine A (CSA). They studied the inhibition of spontaneous cytotoxicity of C57BL/6 and NZB/WF1 mouse spleen cells obtained from cyclosporine A administered mice against YAC and K562 target cells. They reported that CSA inhibited NK cell activity depending upon the dose, but did not require the presence of T cells, B cells or macrophages. It depressed NK activity by direct interaction rather than acting on suppressor cells. The results suggested that the inhibitory properties of CSA can be used as a tool in analysis of the mechanism of spontaneous cytotoxicity.

Fukui *et al.* (1987) examined the natural killer cell activity in the rat, by regarding lymphocytes as effector cells in NK and ADCC activities. They observed an appreciable ADCC activity in peripheral blood leucocytes (PBL), splenic leucocytes (SPL), and peritoneal exudate cells (PEC), but not in cell preparations from the thymus and peripheral lymph nodes. The ADCC activity, when compared with natural killer (NK) activity, was significantly higher in PBL but the same or lower in SPL and PEC.

Lillehoj and Chai (1988) compared the NK cell activities of thymic, bursal, spleenic and intestinal intraepithelial lymphocytes of chickens. They noted a great variability in cytotoxic potential among NK cells of different lymphoid organs. The NK cell cytotoxicity varied depending upon the type of effector cells, type of target cells, and the ratio of effector to target cells, and the age and genetic background of chickens. They noticed substantial levels of NK cell activity in spleen and gut intraepithelial lymphocytes (IEL) of chickens while the effects of target cell specificity demonstrated by NK cells from all the four lymphoid organs were similar.

The level of cytotoxicity was increased with incubation time. Thymus and bursa NK cell activity was not detected in a 4 h assay but substantial NK cell activity was demonstrated in a 16 h assay.

Suresh and Vasudevan (1994) reported that *Emblica officinalis* enhanced natural Killer (NK) cell activity and antibody dependent cell Mediated cytotoxicity (ADCC) in mice bearing Dalton's lymphoma ascites tumor. The results revealed that the antitumor activity of *E. officinalis* was mediated primarily through the ability of the drug to augment natural cell-mediated cytotoxicity.

Lessard *et al.* (1995) studied the cell-mediated and humoral immune responses in chickens infected with *Salmonella typhimurium*. They examined the lymphocyte proliferative responses and NK cell cytotoxicity in 4 weeks old leghorn and New Hampshire after 8 and 20 days of infection with *S. typhimurium* orally. They reported that the lymphocyte proliferative responses and cytotoxic activity of NK cells were significantly higher in infected chickens than in uninfected birds of the same breeds on these days. The results showed that the cellular immunity was activated in the first 3 weeks following infection of 4-weeks- old chickens with *S. typhimurium*.

Won and Lin (1995) reported that the thermal stresses reduce natural killer (NK) cell cytotoxicity. They found that the spleenic NK cell activity began to fall after first day of cold (4^0C) or heat (35^0C) stress and it was more evident after a period of 16 days exposure to heat/cold stress. They also noted rise in adrenal cortisol in both groups.

Ramnath *et al.* (2006) studied the effect of abrin on CMI responses in mice. They noticed that abrin enhanced the proliferation of splenocytes in response to mitogens (PHA 2.5μg and 5 μg , Con A 5μg and 10μg), NK cell activity in both the normal (49.8 per cent cell lysis) and the tumor-bearing group (51.7 per cent cell lysis) on 9th day which was earlier than control group (20 per cent cell lysis on day 11).

They also reported that the ADCC and antibody dependent complement mediated cytotoxicity (ACC) responses were higher in tumor-bearing mice (44 per cent cell lysis on 9th day and 27.6 per cent dead cells by day 15) administered with abrin, when compared to control group (15 per cent cell lysis on 13th day and 14 per cent dead cells on 19th day). Results suggested that intraperitoneal administration of abrin stimulate the CMI responses significantly in tumor-bearing mice.

Thejass and Kuttan (2006) reported the augmentation of natural killer cell and antibody-dependent cellular cytotoxicity in Balb/c mice by sulforaphane, a naturally occurring isothiocyanate from broccoli through enhanced production of cytokines IL-2 and IFN-γ. They studied the effect of sulforaphane in normal and Ehrlich ascites tumor-bearing Balb/c mice and noted that a significant enhancement of NK cell activity and ADCC in both groups as well as an early NK cell activity and ADCC in sulforaphane- treated normal and tumor –bearing animals. Thus the results indicated the enhancement of cell mediated immune response by sulforaphane in mice.

Manu and Kuttan (2007) studied the effect of Punarnavine, an alkaloid from *Boerhaavia diffusa,* on CMI responses using B16F-10, K-562 cell lines and C57 BC/6 mice. They found that the metastatic tumour bearing mice treated with 5 doses of punarnavine (1mg/dose/animal) showed enhanced NK cell activity and the peak NK cell activity was observed on the 5th day (32.4 per cent cell lysis of B16F-10) compared to untreated metastatic control where the maximum cell lysis was only 6.98 per cent on day 9. They also reported that the ADCC and ACC were observed to be enhanced and much earlier in treated animals than in controls. The peak value of ADCC was observed on day 11 (41.5 per cent cell lysis of B16F-10) and the maximum ACC mediated cell lysis of 26 per cent on day 15 of tumor induction whereas, ADCC activity and ACC mediated cell lysis were maximum on 15th day (10.64 per cent and 14 per cent cell lysis respectively B16F-10) in control group. They concluded that punarnavine administration can enhance the cell mediated immunity in tumor bearing mice.

Guruvayoorappan and Kuttan (2007) studied the effect of *Biophytum sensitivum* on the cell mediated immune response in mice. The NK cell activity was increased in normal (43.6 per cent cell lysis of human leukemic cells- K562 and tumor bearing mice 48.2 per cent cell lysis) administered with *B.sensitivum* on day 5 compared to control group on day 9 (13.4 per cent cell lysis of K562 cells). The ADCC is also enhanced in groups given with *B. sensitivum,* the peak activity being 35 per cent cell lysis in non tumor bearing mice and 40.2 per cent cell lysis of sheep red blood cells (SRBCs) in tumor bearing mice on day 7. In untreated tumor bearing mice the peak lysis was on day 11 (12.13 per cent). The maximum ACC mediated cell lysis of Ehrlich`s ascites carcinoma (EAC) cells was observed earlier (15th day) and enhanced as 26.6 per cent in tumor bearing group and 22.6 per cent in normal control group treated with *B. sensitivum* when compared to tumor bearing untreated mice (11.4 per cent cell lysis on 17th day).

Sheeja and Kuttan (2007) reported that the modulation of NK cell activity, ADCC, and ACC by andrographolide in normal and Ehrlich ascites carcinoma-bearing mice. They studied the effect of *Andrographis paniculata* extract (APE) and its isolated compound andrographolide (ANDLE) on cell mediated immune responses in normal and tumor- bearing control mice. Treatment with APE and ANDLE significantly enhanced NK cell activity in normal (46.82 per cent and 40.79 per cent cell lysis) and tumor-bearing animals (48.66 per cent and 42.19 per cent cell lysis) on the fifth day. There was earlier than in tumor-bearing control animals (12.89 per cent cell lysis on day 9). The ADCC was also increased in APE (45.17 per cent cell lysis on day 11) - treated normal and tumor-bearing animals (APE, 47.39 per cent cell lysis; ANDLE, 41.48 per cent cell lysis on day 11) when compared to untreated tumor-bearing control animals (maximum of 11.76 per cent cell lysis on day 17). They also found out that an early enhancement of ACC by admimistration of APE and ANDLE in normal as well as tumor-bearing animals. The results of the study

confirmed that the andrographolide enhanced the cell mediated immune responses in normal and tumor-bearing control animals.

2.8 PROPERTIES OF MAJOR INGREDIENTS OF BRAHMA RASAYANA (BR)

Xia *et al.* (1997) quoted that there were 17 countries in the world that use various parts of *Emblica officinalis* in their medical treatment. The plant was found good in the regulation of gastric function and possessed hepatoprotective, anticancer and anti-inflammatory activities. It was regarded as a traditional immunomodulator and a natural adaptogen.

Kumar *et al.* (1999) studied the effect of "rasayanas" herbal drug preparation on cell-mediated immune response in tumor bearing mice. They found out that the administration of rasayanas enhanced the NK cell activity. The BR was found to enhance ADCC and ACC mediated tumor cell lysis. The results of these studies indicated the usefulness of rasayanas for immunostimulation in normal and in diseased state.

Rekha *et al.* (2000) found that oral administration of BR @ 10 and 50 mg per dose /animal) significantly increased the total leukocyte count and percentage of polymorphonuclear cells in mouse. The bone marrow cellularity as well as alpha esterase positive cells were also increased.

Gowri *et al.* (2001) examined the beneficial influence of *E. officinalis* fruits on iron bioavailability, by virtue of their high ascorbic acid content. They studied four cereals, four pulses, and four combinations of cereals and pulses for the effect of *E. officinalis* fruits included at two concentrations on the *in vitro* iron availability and noticed that supplementation of cereals and pulses with E. officinalis did not seem to have any advantage in terms of iron bioavailability and concluded that tannin content hampered the beneficial effects of ascorbic acid and that the tannin content is inversely proportional to haematinic effect of *E. officinalis* fruits.

Rekha *et al.* (2001a) studied the effect of BR on antioxidant systems and cytokine levels in mice during cyclophosphamide administration. They evaluated the effect of intra peritoneal administration of cyclophosphamide (CTX) 25 mg/kg bw/mouse for 10 days on levels of reduced glutathione (GSH), blood glutathione peroxidase (GPX) and tissue levels of superoxide dismutase (SOD) and catalase (CAT) and found to be suppressed whereas tissue levels of glutathione reductase (GR) and glutathione-transferase (GST) were unaltered while serum and tissue lipid peroxide levels were significantly increased by CTX treatment. They reported that oral administration of brahma rasayana (BR) (50 mg/mouse for 10 days) significantly enhanced the tissue levels of SOD, CAT, GST, GPX, serum and tissue GSH and reduced the serum and tissue lipid peroxidation. It was also noticed that BR administration enhanced the serum cytokine level of interferon-gamma, interleukin-2 and granulocyte macrophage-colony stimulating factor in normal and CTX treated mice. The results indicated that BR reduced the oxidant stress induced by CTX treatment and its effect in cellular function.

Rekha *et al.* (2001b) evaluated the effect of BR on antioxidant system after radiation. They found out that the oral administration of BR (50 mg/ animal for 10 and 30 days) significantly increased the liver antioxidant enzymes such as superoxide dismutase, catalase and tissue and serum reduced glutathione levels also BR administration significantly elevated the suppressed levels of these enzymes due to irradiation. They also noted that BR treatment reduced the serum and liver lipid peroxides which were increased on radiation exposure. Thus the results indicated that BR could ameliorate the oxidative damage produced in the body by radiation and useful as an adjuvant during radiation therapy.

Khandelwal *et al.* (2002) estimated the efficacy of *E. officinalis* in modifying the acute cytotoxicity of Cadmium (Cd) in male rats. Oral administration of *Emblica* fruit juice (500 mg/kg bw) for 8 days followed by a single toxic dose of Cd as $CdCl_3$ (3 mg/ kg bw,i.p) considerably reduced the mortality in rats as well as prevented Cd

induced histopathological damage in testis, liver and kidneys to some extent. They concluded that *Emblica* fruit as a whole comprising considerable amount of Vit.C, tannins, cellulose, amino acids, minerals, metals and cytokine like substances which induced endogenous antioxidant defense system and reduced lipid peroxidation by Cd in target organs.

Sairam *et al.* (2003) revealed the cytoprotective and immunomodulating properties of *E. officinalis* against chromium induced oxidative damage. Gooseberry resulted in enhanced cell survival, decreased free radical production and higher antioxidant levels. It restored both phagocytosis and gamma interferon production by macrophages upon oxidative damage.

Gaddipati *et al.* (2004) evaluated the protective effect of a polyherbal preparation, BR against tumor growth and lung metastasis in rat prostate model. They found out that BR treatment resulted in a 25-37 per cent decrease in palpable tumor incidence, a delay of 1-2 weeks in the tumor occurrence, lower mean tumor volumes, by 14-35 per cent and significant reduction in tumor weight and lung metastasis in comparison to untreated controls. They concluded that the ayurvedic polyherbal preparation, BR played a beneficial role in preventing tumor incidence, tumor growth and metastatic spread.

Na *et al.* (2004) reported that the ethanol extract from the fruit of *Terminalia chebula* exhibited significant inhibitory activity on oxidative stress and the age-dependent shortening of the telomeric DNA length. The *T. chebula* extract exhibited a significant cytoprotective effect against induced oxidative damage. These observations were attributed to the inhibitory effect of the *T. chebula* extract on the age dependent shortening of the telomere.

Naik *et al.* (2004) tested *T. chebula* for potential antioxidant activity by examining its ability to inhibit γ-radiation-induced lipid peroxidation in rat liver microsomes and damage to superoxide dismutase enzyme in rat liver mitochondria

and concluded that it was able to protect cellular organelles from the radiation-induced damage, it may be considered as a probable radioprotector.

Suchalatha and Shyamaladevi (2004) examined the cardioprotective effect of ethanolic extract of *T. chebula* fruits in isoproterenol (200 mg/kg bw) induced myocardial damage in rats. *T. chebula* extract (500 mg/kg bw) pretreatment was found to ameliorate the effect of isoproterenol on lipid peroxide formation and retained the activities of the diagnostic marker enzymes.

Gandhi and Nair (2005) evaluated the aqueous extract of the fruit of *T. chebula* (TCE) for its antioxidant and radioprotective abilities. The TCE (50 μg) was able to neutralize 1, 1-diphenyl-2-picrylhydrazyl, a stable free radical by 92.9%. The free radical neutralizing ability of TCE was comparable to that of ascorbate (100 μ*M*) by 93.5% and gallic acid (100 μ*M*) by 91.5% and was higher than that of the diethyldithiocarbamate (200 μ*M*) which was 55.4%, suggesting the free radical scavenging activity of TCE. The TCE also protected the human lymphocytes from undergoing the gamma radiation-induced damage to DNA.

Lee *et al.* (2005) evaluated the protective effects of an aqueous extract of fruit of *T. chebula* fruit on the tert-butyl hydroperoxide (t-BHP) induced oxidative injury observed in cultured rat primary hepatocytes and rat liver. Both treatment and pretreatment of hepatocytes with the TCE significantly reversed the t-BHP induced cell cytotoxicity and lactate dehydrogenase leakage. The *in vivo* study showed that pretreatment with TCE (500 or 1000 mg/kg) by gavage for 5 days before a single dose of t-BHP (0.1 m*M*/kg. i.p.) significantly reduced the indicators of oxidative stress in the liver and lipid peroxidation, in a dose-dependent manner. Based on these results they speculated that TCE has a potential role to play in the hepatic prevention of oxidative damage in living system.

Srikumar *et al.* (2005) assessed the immunomodulatory activities of Triphala (*T. chebula, Terminalia belerica* and *E. officinalis)* by testing the various neutrophil

functions. Noise (100 dB) stress for 4 h/d for 15 days, was employed to alter the neutrophil functions. Upon exposure to the noise-stress, the neutrophil functions were significantly suppressed and followed by a significant increase in the corticosterone levels. Oral administration of Triphala (1 g/kg/day for 48 days) appeared to stimulate the neutrophil functions and prevented stress induced suppression in the neutrophil functions and also noticed a significant decrease in corticosterone level.

Scartezzini *et al.* (2006) determined ascorbic acid in *Emblica* fruit and particularly in *Emblica* fruit processed according to the Ayurvedic method and showed that the *Emblica* fruit contains ascorbic acid (0.4% $^{w}/_{w}$), and that the Ayurvedic method of processing increased the higher antioxidant activity and a higher content of ascorbic acid (1.28% $^{w}/_{w}$) and concluded that Vitamin C accounts for approximately 45-70% of the antioxidant activity.

Ramnath *et al.* (2008) studied the amelioration of heat stress induced disturbances of antioxidant defense system in chicken by BR. They administered BR to chicken at the rate of 2g/kg bw daily for 10 days prior to and during heat stress (at 40 ± 1^{0}C and relative humidity 80 ± 5 per cent in an environmental chamber) for 4 h daily for 5 or 10 days. They found that a significant increase in the activities of antioxidant enzymes like catalase, superoxide dismutase, liver catalase, glutathione peroxidase and glutathione reductase in BR treated stressed birds compared with untreated controls. They also noticed that the serum and liver reduced glutathione concentration were increased in BR treated stressed and unstressed birds.

Materials and Methods

3. MATERIALS AND METHODS

3.1 CHEMICALS, INSTRUMENTS AND REAGENTS

All the chemicals and reagents used were of analytical quality.

3.1.1 Important chemicals

Chemical		Source
Dulbeco's Modified Eagle Medium (DMEM)	-	Hi media laboratories, Mumbai
Eagle's Minimum Essential Medium (MEM)	-	,,
Rosewell Park Memorial Institute Medium (RPMI-1640)	-	,,
Hanks Balanced Salt Solution (HBSS)	-	,,
Fluid thioglycollate medium	-	,,
Trypsin	-	,,
Sodium caseinate	-	,,
Sodium diatrizoate (Hypaque)	-	,,
Lactalbumin hydrolysate	-	,,
Yeast powder	-	,,
Corticosterone standard	-	,,
Concanavalin A (Con A)	-	Sigma Chemicals Company, USA.
Bovine serum albumin (BSA)	-	,,
Dioxan	-	,,
Agarose	-	,,
PPO (2,5-Diphenyl oxazole)	-	,,
Geimsa stain solution	-	Sisco Research Laboratories, Mumbai.
POPOP (1,4-bis-2-5-phenyl oxazolyl benzene)	-	,,
Foetal calf serum (FCS)	-	Biological Industries, Kibbutz, Beit Haemek, Israel.
^3H Thymidine	-	Board of Radiation and Isotope Technology (BRIT), Bhaba Atomic Research Centre (BARC), Mumbai.
Sodium chromate ($Na_2{}^{51}CrO_4$) (Specific Activity > 50 mci/mg)	-	,,

Methyl Thiazolyl diphenyl tetrazolium bromide – blue(MTT)	-	Sd fine chemicals Ltd, Mumbai.
Dimethyl Sulphoxide (DMSO)	-	”
L-Glutamine	-	”
Trypan blue	-	Nice Reagents, Kochi, Kerala.
Ficoll	-	Genei, Bangalore.
Methylene chloride	-	Merck Specialities, Pvt Ltd, Mumbai.
Phytohemagglutinin (PHA)	-	Difco Laboratories, USA

3.1.2 Instruments

Cooling Centrifuge	-	Remi, Chennai.
Deep freezer (- 20 ^{0}C)	-	”
Autoclave	-	”
Gamma ray spectrophotometer	-	Wallac-Wizard.
Inverted microscope	-	Leica, Germany.
Incubator	-	Spinco, Chennai.
Laminar flow hood	-	Klenzaids Contamination, Control Pvt.Ltd, Gujarat.
Liquid Scintillation Counter	-	Rack Beta 1209, LKB Wallac.
Research Microscope	-	Labomed, India.
UV-Vis spectrophotometer	-	Elico Pvt. Ltd, India.
Fluorospectrometer	-	Nanodrop, Wilmington, USA.

3.1.3 Reagents

3.1.3.1 *Phosphate buffer saline (PBS)*

Sodium chloride	-	8.00 g
Potassium chloride	-	0.20 g
Disodium hydrogen phosphate	-	1.44 g
Potassium dihydrogen phosphate	-	0.02 g

Distilled water	-	1000 ml

The pH was adjusted to 7.2 with 1 *N* HCl or NaOH.

3.1.3.2 *PBS-Ethylene diaminetetraaceticacid-dipotassium salt (EDTA) solution*

EDTA	-	20 mg
PBS	-	100 ml

Autoclaved at 10 lbs for 15 min.

3.1.3.3 *Trypsin solution*

Trypsin	-	200 mg
Glucose	-	20 mg
PBS-EDTA	-	100 ml

Sterilized by filtration.

3.1.3.4 *Alsever's solution*

Dextrose	-	2.05 g
Sodium citrate	-	0.80 g
Sodium chloride	-	0.42 g
Distilled water	-	100 ml

The pH was adjusted to 6.1 with 10 per cent citric acid, autoclaved at 10 lbs for 15 min and stored at 4^0C.

3.1.3.5 *Scintillation fluid*

PPO	-	2.5 g
POPOP	-	0.25 g
Naphthalene	-	100 g
Dioxan	-	1000 ml

3.1.3.6 *Stain*

Trypan blue	-	100 mg
Saline (0.9%)	-	100 ml

3.1.3.7 ***Fixative***

Methanol	-	70 ml
Acetic acid	-	10 ml
Distilled water	-	20 ml

3.1.3.8 ***L- glutamine stock solution***

L-glutamine solution with a concentration of 200 m*M* was prepared by dissolving 29.23 g L-glutamine powder in one litre of double distilled water. Then filtered using Millipore filter (0.2 μm) into 10 ml aliquots and stored at 4^0C until use. The glutamine stock solution was again diluted to 1:100 with double distilled water to get a concentration of 2 m*M*.

3.1.3.9 ***Solution A for differential centrifugation***

Ficoll	-	9 g
Distilled water	-	100 ml

3.1.3.10 ***Solution B for differential centrifugation***

Hypaque	-	33 g
Distilled water	-	100 ml

3.1.3.11 ***Solution C for differential centrifugation***

Hypaque	-	50 g
Distilled water	-	100 ml

3.1.3.12 ***Ficoll - hypaque solutions of different specific gravities***

Mixed 24 parts of solution A and 10 parts of solution B to get a solution having specific gravity 1.077 and mixed 20 parts of solution A and 10 parts of solution C to get a specific gravity 1.119.

3.1.4 Sterilization of glassware

All glassware and filtration apparatus used for tissue culture purposes were soaked in the solution of Extran (one per cent) overnight, cleaned using brush and washed thoroughly under running water. All the glassware were rinsed in distilled water and dried in a hot air oven. Autoclaved at 15 lbs / square inch for 15 min, dried and used for experiments.

3.1.5 Preparation of culture media

Using autoclaved double distilled water, DMEM (9.98 g/l), MEM (10.3 g/l) and RPMI-1640 (10.3 g/l) were prepared; pH was adjusted to 7.2 and filtered under negative pressure using a 0.22 µm cellulose filter assembly. Sterility of the medium was tested using fluid thioglycollate (@ 929.8 g/l) medium. For this, 10 ml sterile thioglycollate medium was inoculated with 1 ml of medium prepared, incubated at 37^0C for 6 days, and checked for visible contamination. Antibiotics such as Benzyl penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added to the medium prior to use.

3.1.6 Cell lines

Mouse lung fibroblast (L929) cells and Human leukaemic (K562) cells were obtained from National Centre for Cell Sciences, Pune, India. Ehrlich ascites carcinoma (EAC) cells were obtained from Cancer Research Institute, Mumbai and these were maintained as ascites tumour in Swiss albino mice.

3.1.6.1 *Maintenance of cell lines*

3.1.6.1.1 *L929 cells*

The spent medium was removed from the confluent bottles and the cells were washed thrice with two ml of PBS-EDTA. One ml of trypsin solution containing 0.02 per cent EDTA was added and incubated for 3-4 min at 37^0C and the bottles were tapped to dislodge cells. To this, MEM (5 ml) containing 10 per cent goat

serum and antibiotics (complete medium) was added. Cells were dispersed to a single cell suspension by repeated pipetting and an aliquot of cell suspension was added to fresh cell culture bottles containing 10 ml of complete medium and incubated at 37^{0}C. Cells were subcultured every week.

On the day of the experiment, the spent medium was removed, monolayer was washed thrice with PBS and the cells were layered with one ml of trypsin containing EDTA and incubated at 37^{0}C for 3-4 min. Cells were dislodged by tapping the bottles and a single cell suspension was prepared by adding 5 ml MEM having 10 per cent goat serum and pipetted repeatedly. Cell viability was checked by trypan blue dye exclusion method (Kuttan and Kuttan, 1992).

3.1.6.1.2 ***K562 cells***

The cell suspension was mixed well and dispersed the clumps by repeated micropipetting in order to make a single cell suspension. The cells were counted and 1×10^6 cells were seeded to fresh bottles containing 10 ml of RPMI-1640 medium with 10 per cent FCS, antibiotics and incubated at 37^{0}C and sub cultured every third day.

On the day of the experiment, single cell suspension of K562 was prepared. The cells were counted, viability was checked and concentration was adjusted with RPMI-1640 medium containing 10 per cent FCS and antibiotics.

3.2 BIRDS

A total of 144 male day-old, egg type local strain: Gramapriya (*Gallus domesticus*) were procured from Kerala Agricultural University Poultry Farm, Mannuthy, Thrissur and were reared in a battery brooder under standard managemental conditions. As per the feeding recommendation of Bureau of Indian Standards (BIS)-1992, New Delhi, India, they were fed with a commercial starter ration for the first four weeks and at the end of the first month, chicks were fed with commercial adult layer mash till they attained 1 kg body weight (bw). Birds were fed

in the morning and had free access to water *ad lib.* Permission of the Institutional Animal Ethics Committee was obtained for the conduct of the study.

3.3 HEAT STRESS (HST) / COLD STRESS (CST) REGIME

The protocol followed in this study was a combination of two earlier reports (Thaxton 1978 and Kulkarni and Varshney 2005). A controlled environmental unit with a holding capacity of 12 birds at a time in 6 chambers (area 1800 cm^2/chamber), with provisions for adjusting humidity and temperature was used. Chamber temperature of 40 ± 1^{0}C and relative humidity (RH) of 80 ± 5 per cent were chosen for the present study. To the above conditions chickens were exposed for 4 consecutive hours per day for 10 days. During the period of exposure to HST, chickens were provided with neither drinking water nor feed. Chamber temperature of 4 ± 1^{0}C and relative humidity (RH) of 45 ± 5 per cent were chosen as CST regime for the present study. To the above conditions chickens were exposed for 4 consecutive hours per day for 10 days and during the period of exposure to CST, chickens were provided with neither drinking water nor feed.

3.4 DRUG

3.4.1 Composition of Brahma Rasayana (BR)

Name of the ingredient	Per cent	Name of the ingredient	Per cent
1.*Urarira pitca*	0.40	20.*Cinnamomum iners*	0.16
2. *Desmodium gangeticum*	0.40	21.*Elettaria cardamomum*	0.16
3.*Gmelina arborea*	0.40	22.*Cyperus rotundus*	0.16
4. *Solanum nigrum*	0.04	23.*Curcuma longa*	0.16
5.*Tribulus terrestris*	0.40	24. *Aquilaria agallocha*	0.16
6. *Aegle marmelos*	0.40	25. *Piper longum*	0.16

7. *Premna tomentosa*	0.40	26. *Santalum album*	0.16
8. *Stereospermum suvaeolens*	0.40	27. *Clitoria ternate*	0.16
9. *Sida rhombilfolia*	0.40	28. *Centella asciatica*	0.16
10. *Boerhaavia diffusa*	0.40	29. *Mesua ferrea*	0.16
11. *Ricinus communis*	0.40	30. *Acorus calamus*	0.16
12. *Vigna vexilata*	0.40	31. *Scirpus crossus*	0.16
13. *Phaseolus adenanthus*	0.40	32.*Glycyrrhiza glabra*	0.16
14. *Asperagus racemosus*	0.40	33. *Embelia ribes*	0.16
15. *Holostemma annulare*	0.40	34. *Emblica officinalis*	20.00
16. *Leptadenia reticulate*	0.40	35. *Terminalia chebula*	6.67
17. *Desmostachya bipinnata*	0.40		
18. *Saccharum officinarum*	0.40		
19. *Oryza malampuzhensis*	0.40		

3.4.2 Method of preparation of BR

The BR was purchased from Oushadhi, the Pharmaceutical Corporation (I.M), a company under Health and Family Welfare Department, Government of Kerala, Thrissur, which was prepared as per standard recommended procedures (Anonymous 1978 and Sharma 1987). The method of preparation included: 19 items from *Urarira pitca* to *Oryza malampuzhensis* were cut into small pieces and washed well. Water (16 parts) was added to the total quantity of drugs and allowed to boil to obtain one fourth of the original volume. The seeds from fruit of *Emblica officinalis* and *Terminalia chebula* were removed and the pulp was roasted (60^0C) by adding

sufficient quantity of ghee and sesame oil. Then a sufficient quantity of sugar was added to the above decoction to yield a paste-like form. The other 14 items from *Cinnamomum iners* to *Embelia ribes* were cleaned and dried well under shade and made into a fine powder that was then mixed with the above paste. Sufficient quantity of honey was added (when the preparation came to normal temperature), mixed well and stored at room temperature.

3.4.3 Administration of BR

As a pre-trial test, oral administration of BR at various dose levels ranging from 0.5 to 6.0 g/kg bw of bird, daily for 20 days was tested and found that it did not induce any drug-related toxicity. No significant weight loss or gain was observed, reflecting the effect of BR administration on the well being of birds. Even at the highest concentration of 6.0 g/kg bw, the birds showed no visible toxicity as measured by loss of appetite, lack of movement and alertness. Therefore it was concluded that BR is a non-toxic herbal preparation since its administration did not influence weekly gain of body weight of treated birds. However, for all experiments the dose used was 2.0 g/kg bw which was in par with the dose administered to mice *i.e.* 50 mg per mouse of 25 g bw (Rekha *et al.*, 2000). The required quantity of BR was made into suspension using warm water (5 parts) and mixed with about 30 g of poultry feed (one-third of total daily ration) and fed to experimental birds as the daily first meal, followed by heat / cold exposure. Measures to determine wastage of drug mixed feed were undertaken.

3.5 EXPERIMENTAL DESIGN FOR CELL MEDIATED IMMUNE RESPONSE I

Three sets of trials were carried out using 144 birds in this study. For the first trial a total of 48 birds (4 months age) were utilized and divided into 6 groups (G I to G VI) comprising of 8 birds/group.

G I: Non- heat / cold stressed and untreated birds. They were reared randomly under ambient temperature of 30 ± 1^{0}C and 65 per cent RH.

G II: Non- heat / cold stressed and BR treated (2 g/kg bw orally) for 20 days. Birds were reared under similar conditions of temperature and humidity as G I.

G III: HST at 40 ± 1^{0}C and RH of 80 ± 5 per cent for 4 consecutive h/day for 10 days and untreated.

G IV: HST at 40 ± 1^{0}C and RH of 80 ± 5 per cent for 4 consecutive h/day for 10 days and BR treated (2 g/kg bw orally) for 20 days (10 days prior to and 10 days during the experimental period of heat exposure).

G V: CST at 4 ± 1^{0}C and RH of 45 ± 5 per cent for 4 consecutive h/day for 10 days and untreated.

G VI: CST at 4 ± 1^{0}C and RH of 45 ± 5 per cent for 4 consecutive h/day for 10 days and BR treated (2 g/kg bw orally) for 20 days (10 days prior to and 10 days during the experimental period of cold exposure).

3.6 ASSESSMENT OF HEAT / COLD STRESS

3.6.1 Body temperature and hydration level

Rectal temperature of experimental birds was recorded before starting the experiment and 4 h after heat / cold treatment. The initial and final body weights of birds was noted before and after heat / cold exposure and the differences in weight were calculated to find out the hydration status of the body.

3.6.2 Estimation of plasma corticosterone

On day 5, after heat / cold exposure, 2 ml blood was collected by wing vein puncture in heparinised vials from birds of G III, G IV, G V and GVI and centrifuged at 2000 rpm for 20 min to get plasma. The plasma corticosterone level was estimated using the method of Mattingly, 1962. One ml of plasma was washed with 3 volumes of petroleum ether by shaking vigorously for 30 s in a capped test tube. After centrifugation, the solvent layer was discarded. One ml of the washed sample was diluted with 2.5 ml distilled water and was extracted by shaking 30 s with 7.5 ml of

methylene chloride. After centrifugation (2000 rpm), the bulk of the aqueous phase was carefully removed by aspiration and discarded, and 1 ml of 0.1*N* NaOH was added to the tube containing the solvent extract. The tube and contents were quickly shaken for 10-15 s and centrifuged (2000 rpm), the alkaline wash was discarded, and 2 ml of 30*N* sulfuric acid was added to the tubes carefully along the sides.

The tubes were capped and shaken for 30 s with the thumb tightly over the cap. The pressure was released carefully, protecting the eyes and clothing from the resultant acidic spray and after centrifugation (2000 rpm), for 2 min the supernatant solvent layer was discarded by aspiration.

After holding 60 min at room temperature, the fluorescence of the acid layer of the samples were read at excitation 470 nm and emission 530 nm in fluorescent spectrometer. Earlier a standard curve was constructed using corticosterone (0.01-10 µg) and used for referring relative fluorescence units (RFU) of test samples against the standard curve. Corticosterone levels of birds of G III to G VI were then compared with G I and G II. Corticosterone levels of birds of G I and G II were also estimated in a similar way.

3.7 CELL MEDIATED IMMUNE RESPONSES THROUGH MACROPHAGES

3.7.1 Preparation of peritoneal macrophages

Peritoneal macrophages were elicited by injecting 10 ml of five per cent sodium caseinate solution i.p. in all groups of birds 48 h before the completion of experiment. After two days, birds were sacrificed by cervical dislocation. The feathers were removed and injected aseptically with 50 ml of cold PBS i.p. The peritoneal cavity was gently prodded and the peritoneal fluid containing macrophages was aspirated. The cells were washed thrice with PBS and suspended in RPMI-1640 to the desired cell concentrations.

3.7.2 Determination of cell viability

Cell viability was determined by trypan blue dye exclusion method (Kuttan and Kuttan, 1992). Cell suspension (900 µl) was mixed with 100 µl of 1 per cent trypan blue, kept for 2-3 min and loaded on a haemocytometer. Viable cells excluded trypan blue dye, while non-viable cells took up the dye and thus appeared blue in colour. The number of stained and unstained cells was counted separately.

$$\text{Per cent dead cells} = \frac{\text{Number of dead cells}}{\text{Number of viable cells} + \text{Number of dead cells.}} \times 100$$

3.7.3 Macrophage culture

To establish macrophage monolayer, 1×10^6 live peritoneal macrophage suspension/ml from each bird was added to a 35 mm petri dish containing 4 sterile glass cover slips. These were incubated for 1 h at 41^0C, in 5 per cent CO_2 atmosphere to facilitate cell adherence. Three of the four cover slips were washed with 0.85 per cent sterile saline to remove any nonadherent cells. Then they were transferred to new petri dishes containing fresh RPMI-1640 growth medium with 5 per cent FCS.

3.7.4 Determination of phagocytic activity of macrophages

The phagocytic potential of adherent macrophages was determined by sheep red blood cells (SRBCs) phagocytosis assay using opsonised SRBC with rabbit anti SRBC antibody (Mehra and Vaidya, 1993).

Sheep (Ramnad white breed) reared in Goat and sheep farm, College of Veterinary and Animal Sciences, Kerala Agricultural University, Mannuthy, was used for blood collection. About 10 ml of venous blood was collected in equal volume of Alsever's solution, and stored at 4^0C till further use.

Anti SRBC antibody was prepared in a young healthy Newzealand white male rabbit (4 months old), by injecting 0.5 ml of 20 per cent trypsinized SRBC in saline intradermally into the dorsal skin. A booster dose was given four weeks after the initial dose. Next day after the booster dose, animal was bled and serum was

separated; heat inactivated at 56^0C for 30 min and checked the antibody titre by the hemagglutination (HA) assay.

The HA assay was performed as follows. The rabbit serum (100 μl) was serially diluted with PBS in U- bottomed 96 well microtitre plate. An equal volume of two per cent erythrocyte suspension was then added to each well and incubated for one hour at room temperature. The degree of agglutination was evaluated macroscopically and the HA titre was calculated as the reciprocal of highest dilution of serum that showed visible agglutination. The serum was diluted to get the titre of 1:50.

Sheep blood was washed with PBS repeatedly to retrieve SRBCs and two per cent of the solution was prepared. Equal volumes of SRBC and rabbit anti SRBC antibody were mixed and kept at 37^0C, for 1 h, followed by three washings with PBS. The opsonized SRBC solution was layered over the cover slips carrying adherent macrophages and incubated for 1 h at 37^0C. Final washing with PBS was done to remove non bound SRBCs. Cover slips were stained with Geimsa. Out of 200 cells counted, the number of macrophages with opsonised SRBC antibody complex inside was noted.

3.7.5 Determination of cytotoxic activity of macrophages

To find out the cytotoxicity potential, peritoneal macrophages were collected from the birds as described in 3.7.1. Macrophages at a concentration of $2.5x10^3$ per 100 μl were added to each of 96 well flat bottom titre plates, in triplicate for each group of birds and incubated for 24 h in 5 per cent CO_2 atmosphere at 37^0C. After 24 h, L929 cells ($5x10^3$) were added to each well containing macrophages. The final volume was adjusted to 250 μl with RPMI-1640 complete medium and incubated for another 48 h. The control wells were maintained in triplicate containing $5x10^3$ L929 cells alone.

Twenty μl of MTT (5 mg/ml) was added 4 h before the completion of incubation (Cole, 1986 and Campling *et al.*, 1991). The titre plate was centrifuged at

1500 rpm for 15 min. Medium along with reagents were aspirated and 50 µl of 98 per cent DMSO was added to each well and the plate was shaken slowly for 15 min for colour development. The absorbance was measured spectrophotometrically at 570 nm. The percentage cytotoxicity was calculated using optical density (OD) values of all groups compared with control wells.

3.7.6 Determination of macrophage migration index (MMI)

Macrophage migration index was calculated as per the procedure of Saxena *et al.* (1991), with certain modifications as described by Sujarani (2003). Peritoneal macrophages were elicited with sodium caseinate as mentioned in 3.7.1. Batches with 90 per cent or more viability were used for the test. The cells were finally suspended in 0.5 ml RPMI-1640 medium containing antibiotics, 2 m*M* glutamine and 10 per cent heat inactivated FCS. Cells were counted in hemocytometer using WBC diluting fluid. The final cell suspension was adjusted to a concentration of 80×10^6 cells/ml of medium.

The peritoneal macrophages thus prepared from treated / untreated and stressed / non-stressed birds were packed in the microhaematocrit capillary tubes of uniform diameter, and were placed in migration chambers (24 well flat bottom micro titre plate), filled with complete RPMI-1640 medium containing five per cent FCS, antibiotics and glutamine and then incubated at 37^0C for 18-24 h.

At the end of incubation period, the cells were fixed to the surface by flooding the plates with methanol acetic acid fixative for 15 min. The migration area of macrophage was measured by taking the average diameter of the opaque zone around the capillary.

The migration area of macrophages from the heat / cold stressed group divided by that of non-stressed control birds was expressed as macrophage migration index (MMI).

3.8 CUTANEOUS DELAYED HYPERSENSITIVITY RESPONSE

To determine the chicken's ability to display a cell - mediated response to a mitogen, cutaneous delayed hypersensitivity responses were carried out using mitogens. The hypersensitivity responses against phytohemagglutinin-P (PHA-P), considered to be a predominant T-cell mitogen, was studied in birds exposed to heat / cold stress as well as in non-stressed controls. As a part of priming procedure, intradermal inoculation of 20 µg of PHA in 100 µl PBS was carried out in the wattles of all cockerels. After 7 days of heat / cold stress, PHA-P (100 µg/100 µl) was injected intradermally to induce the skin reactions, into the left toe web and 100 µl of sterile saline into the right toe web to serve as the control (Kishimoto *et al.*, 1987). Toe web thickness (in mm) was measured at 0, 24, 48 and 72 h post mitogen injection using a vernier caliper. Post mitogen injection leading to progressive toe web swelling was considered to be an indication of cutaneous delayed hypersensitivity response.

3.9 LEUCOCYTE MIGRATION INHIBITION TEST (LMIT)

This was carried out according to the method described by Bendixen (1977) with minor modifications as done by Jayaprakasan (1986) on 10th day of the experiment.

Blood (2 ml) was collected by wing vein puncture in EDTA (dipotassium salt, 1.5 mg/ml) from all groups of birds on 10th day after exposure to temperature stress. Three ml of ficoll-hypaque with specific gravity 1.119 as mentioned in 3.1.3.12 was taken in a test tube and 3 ml of ficoll-hypaque with specific gravity 1.077 was layered over. Anticoagulated blood was mixed with MEM (1:1) and two ml of this solution was layered on the top of the gradient. The test tubes were centrifuged for 45 min at 1500 rpm at room temperature. After centrifugation, the cloudy layer extending from the 1.077 to 1.119 interfaces to the top of the erythrocytes pellet was collected and transferred to another test tube. Then the cell suspension was washed with PBS

containing 0.02 per cent EDTA and centrifuged at 1500 rpm for 10 min. Contaminating erythrocytes were removed by hypotonic lysis by resuspending the cell pellet in one ml of distilled water, agitating briefly, and restoring tonicity by adding four ml of PBS and 0.02 per cent EDTA. This suspension was centrifuged at 1500 rpm for 7 min. Pellet was washed once to remove erythrocytes lysate product. The washed leucocytes were resuspended in normal saline containing approximately $1.5x10^8$ cells per ml.

The agarose medium was prepared fresh on the day of use. Agarose (2 per cent) in distilled water was boiled and cooled to 45^0C, to which equal quantity of double concentration HBSS (2x), of pH 7.2 containing 1 per cent lactalbumin hydrolysate, 0.4 per cent yeast extract, 20 per cent FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) was added. Agarose medium kept at 45^0C was poured into petri dishes to get 3 mm thickness when solidified. The plates were then incubated at 37^0C for 1 h prior to use.

The leucocyte suspension prepared ($1.5x10^8$ cells/ml) was divided into two equal parts and to one portion 100 µl of antigen (BSA) and to the other portion 100 µl of sterile normal saline was added. Contents of each tube were thoroughly mixed and then incubated for 1 h at 37^0C with occasional shaking to avoid cell clumping. Six wells of 3 mm diameter were cut 8 mm apart in the agarose gel, into which the contents of each tube were filled. The charged plates were incubated at 37^0C in a humid chamber for 20 h. At the end of incubation period, the cells were fixed to the glass surface by flooding the plates with methanol acetic acid fixative for 15 min. The gel in the plates was then partially dried to facilitate pealing off agarose from the plates.

Migration area (cm^2) was measured by taking average diameter of the opaque zone around the wells. The migration index was calculated as the average area of migration of cells treated with antigen divided by the average area of migration of cells treated with normal saline.

3.10 SPLENOCYTE PROLIFERATION ASSAY

3.10.1 Preparation of spleen cells

Birds were killed by cervical dislocation on day 10 of exposure to temperature stress. The skin was removed and an incision was made on the left side just below the sternum. The spleen was removed without any adherent tissue, aseptically, into a petri dish containing cold RPMI-1640 medium supplemented with 10 per cent FCS. The spleen was teased with a toothed forceps and gently passed through a sieve, pressing with a plastic plunger of a disposable syringe under the flow of cold HBSS. Cells were washed thrice with HBSS, resuspended in a tube containing cold RPMI-1640 and left at 4^0C for 45 min. Supernatant cells were collected and were washed with the medium containing 10 per cent FCS and viability of the cells was checked by trypan blue dye exclusion method. The single cell suspension of spleen thus prepared was maintained in RPMI-1640 medium containing 10 per cent FCS, antibiotics at a concentration of $1x10^7$ cells/ml till further use. All the procedures were done under sterile condition.

3.10.2 *In vitro* lymphoblastogenesis (splenocyte) response

Principle:

Mitogens can stimulate *in vitro* resting lymphocytes to undergo a series of biochemical and physical changes and are converted to blast cells. This process leads to cell division, which can be quantitated by ^{3}H thymidine uptake method (Mustafa, 1992).

Procedure:

Splenocytes ($5x10^4$) obtained from birds of G I to G VI were incubated in a final volume of 200 µl of RPMI-1640 medium in 96 well flat bottom titre plates supplemented with 10 per cent FCS and antibiotics, in a humidified atmosphere containing 5 per cent CO_2 at 37^0C for 48 h. The mitogens, PHA at concentrations of 2.5 µg/well and 5 µg/well and Con A at concentrations 5 µg/well and 10 µg/well were

added in. One µCi of ^3H thymidine was added to each well and incubated further for 18 h under same conditions. Cultures were centrifuged at 1500 rpm for 10 min. Supernatant was discarded and the pellets were dissolved in 200 µl of 6 *N* NaOH and incubated at 37^0C for 2 h. The contents were transferred to 5 ml scintillation fluid and kept overnight in dark. Radioactivity was measured as counts per minutes (cpm) in a liquid scintillation counter.

3.11 EXPERIMENTAL DESIGN FOR CELL MEDIATED IMMUNE RESPONSE-II

For the second set of trial, a total of 48 birds was utilized and divided into 6 groups (G I to G VI) comprising of 8 birds/group as done in 3.5 with the following modifications. Exposure to heat / cold stress as well as administration of BR was continued for the respective groups till all cockerels were sacrificed from each group. One bird from each group was sacrificed on alternate days, from day 5 onwards and the spleen was utilized for experiments described under 3.12.

3.12 CELL MEDIATED IMMUNE RESPONSES THROUGH LYMPHOCYTES

3.12.1 Natural killer (NK) cells response

3.12.1.1 ***Labelling of cells with Chromium (^{51}Cr)***

Principle:

The ^{51}Cr binds to cytoplasmic proteins after diffusing through the live cell membrane and is released only when the cell membrane is sufficiently damaged.

Procedure:

The target cells; one million K562 and 10 million SRBCs were washed twice with RPMI-1640 and were resuspended in few drops of FCS in 200 µl of medium. About 100 µCi of $Na_2{}^{51}CrO_4$ was added and incubated at 37^0C for 1 h on a shaker. The cells were washed in medium twice and allowed to incubate in large volumes (5 ml) of medium for 1h at 4^0C. Cells were washed twice in medium and resuspended in complete medium at a concentration of $1x10^5$ cells/ml.

3.12.1.2 *Determination of NK cell mediated cytotoxicity*

Principle:

The ^{51}Cr release assay was used to determine the cytotoxicity, mediated by immune effector cells such as NK cells (Perlman *et al.*, 1981). The ^{51}Cr labeled K562 cells act as target cells and spleen cells as the effector cells at effector target ratio of 100:1.

Procedure:

The test was performed in round bottom titre plates as per method of Gupta and Battacharya (1983). Spleen cells (effector cells; 1×10^6 cells /100 µl / well) of all group of birds were mixed separately with ^{51}Cr labeled K562 cells (1×10^4 cells /100 µl / well) (target cells) in micro titre plates. Final volume was adjusted to 250 µl with RPMI-1640 and incubated at 37^{0}C for 4 h. After incubation, titre plates were centrifuged at 1500 rpm for 15 min at 4^{0}C and supernatant (100 µl) was collected in radioimmunoassay tubes. All tests were done in triplicates.

Spleen cells of unstressed / untreated control chicken were utilized for finding spontaneous and total release. Spontaneous release of ^{51}Cr was determined by incubating labeled target cells in the absence of effector cells and total release by incubating the target cells with 1*N* HCl. The activity of released chromium in the supernatant was counted using a gamma ray spectrophotometer and the percentage cell lysis was calculated using the formula.

$$\text{Per cent lysis} = \frac{\text{Experimental release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

3.12.2 Antibody dependent cellular cytotoxicity (ADCC) response

3.12.2.1 *Determination of mimimum haemolytic dose (MHD) for ADCC assay*

The serially diluted anti SRBC antibody (raised in rabbit) in PBS was mixed with an equal volume of two per cent SRBC. A volume of 200 µl of this preparation was incubated with 1:1 diluted fresh rabbit serum at 37^{0}C for 30 min. The tubes were

then centrifuged and optical density of the supernatant was measured spectrophotometrically and the percentage lysis of the sensitized SRBC was determined. Double the minimum haemolytic dose (2 MHD) of anti SRBC antibody was used for ADCC.

3.12.2.2 *Determination of ADCC*

Principle:

The ^{51}Cr release assay was used to determine the cytotoxicity, mediated by immune effector cells such as T lymphocytes expressing Fc (Perlman *et al.*, 1981). The ADCC is performed by incubating ^{51}Cr labeled SRBC as target cells and spleen cells as effector cells in presence of 2 MHD of anti SRBC antibody. The specific lysis is determined by ^{51}Cr release.

Procedure:

The anti SRBC antibody with double MHD was used as the source of antibody in the assay and 50 µl of it was incubated with target cells at 37^{0}C for 4 h. Labeled SRBC coated with antibodies (1×10^4 cells /100 µl / well) was considered as target cells and spleen cells as effector cells (1×10^6 cells /100 µl / well). The effector target ratio was 100:1. The final volume was made up to 250µl with complete medium and the 4 h ^{51}Cr release assay was performed as explained in 3.12.1.2. The cytolysis was calculated as 3.12.1.2.

3.13 EXPERIMENTAL DESIGN FOR CELL MEDIATED IMMUNE RESPONSE – III

For the third set of trial, a total of 48 birds was utilized and divided into 6 groups (G I to G VI) comprising of 8 birds /group as done in 3.5, for the experiment described under 3.14.

3.14 DETERMINATION OF ANTIBODY DEPENDENT COMPLEMENT MEDIATED CYTOTOXICITY (ACC)

Principle:

When tumour cells are incubated with specific antibodies in presence of complement, the classical pathway will be activated leading to the lysis of target cells.

Procedure:

The EAC cell line which was maintained as ascites tumour in Swiss albino mice was drawn aseptically and washed thrice with saline. About $1x10^6$ cells (1 ml) was inoculated to birds of all group i.v. on the first day of experiment. Blood was collected from each bird, on days 5 and 10 of heat / cold exposure. Serum was separated and the heat inactivated (containing anti EAC antibody). It was diluted with RPMI-1640 medium to get 1:1 dilution. The diluted serum (100 µl) was mixed with target cells ($1x10^4$ EAC cells). Fresh rabbit serum (1:10 diluted with medium) was added (50 µl) as a source of complement and the final volume was made up to 2 ml with complete medium and incubated at 37^0C for 3 h. Controls included (a) without antibody or complement, (b) with antibody and without complement and (c) without antibody and with complement. All tests were done in triplicates. Cells were centrifuged and one ml of the supernatant was discarded and cytotoxicity was assessed by trypan blue dye exclusion method.

3.15 STATISTICAL ANALYSIS

Student's t test was employed for comparison of treatments while, one way analysis of variance (ANOVA) followed by least significant difference (LSD) test was adopted for comparison of more than two treatments.

The split on time analysis was used for comparison of treatments at different time periods (Snedecor and Cochran, 1994).

Results

4. RESULTS

4.1 EFFECT OF HEAT / COLD STRESS ON BODY TEMPERATURE AND BODY WEIGHT

Body temperature was found to be significantly ($P<0.05$) increased in G III (heat stressed and untreated) and G IV (heat stressed and BR treated) cockerels when compared to G I (non-heat stressed and untreated) and G II (non-heat stressed and BR treated) control cockerels. In heat stressed cockerels, the body temperature reached 44.0 ± 0.2^0C by the end of 4 h exposure to heat, while control birds recorded rectal temperature of 42.9 ± 0.2^0C. Cockerels exhibited a great deal of stress related behavioural changes such as gular flutter, drooping of wings, prostration and drowsiness during the period of heat stress. The heat stressed (HST) cockerels lost an average of 35 g bw daily, upon 4 h exposure to 40 ± 1^0C and 80 ± 5 per cent RH, which accounted for an average loss of 3.40 percentage of bw.

Body temperature was found to be significantly ($P<0.05$) decreased in G V (cold stressed and untreated) and G VI (cold stressed and BR treated) cockerels when compared to G I and G II. In cold stressed (CST) cockerels, the body temperature became 42.0 ± 0.2^0C by the end of 4 h exposure to cold, while control birds recorded a rectal temperature of 42.9 ± 0.2^0C. Cockerels exhibited a great deal of stress related behavioral changes such as covering unfeathered portions of the body with feathered portions, tucking the head under the wing, huddling and fluffing out the feathers. There was no significant ($P>0.05$) change in bw of CST cockerels during the experiment period.

4.2 EFFECT OF HEAT / COLD STRESS ON PLASMA CORTICOSTERONE LEVEL

Results of the study revealed that the plasma corticosterone concentration in G III increased significantly ($P<0.05$) to 82.50 ± 0.18 µg/ml on day 5 of heat exposure, when compared to all other groups. It was also found that plasma

172850

corticosterone level decreased significantly ($P<0.05$) in G II, G V and G VI birds when compared to G I. Cockerels of G IV also showed difference ($P<0.05$) in plasma corticosterone level when compared to G I (Table 1).

4.3. EFFECT OF HEAT / COLD STRESS ON CELL MEDIATED IMMUNE (CMI) RESPONSES THROUGH MACROPHAGES

4.3.1 Phagocytic activity of macrophages

The results indicated that the per cent of macrophages exhibiting phagocytic activity against opsonised SRBCs were significantly different ($P<0.05$) between groups, except between G IV and G VI. The maximum phagocytic potential was encountered with G IV cockerels (30.75 ± 1.66 per cent) and the least with cockerels of G I (6.00 ± 0.57 per cent) [Table 2, Plate 1(Fig i and ii)].

4.3.2 Cytotoxic activity of macrophages

It was observed that the macrophage mediated cytotoxicity towards L929 cells was 88.00 ± 1.01 per cent in G IV cockerels, which was significantly ($P<0.05$) higher when compared to G III cockerels with 58.50 ± 1.29 per cent. There was significant ($P<0.05$) decrease of cytotoxicity in G V cockerels (62.00 ± 0.57 per cent) when compared with cockerels of G II (84.50 ± 1.12 per cent). The cytotoxic response against L929 cells in G I and G VI birds were almost similar (Table 2).

4.3.3 Macrophage migration index (MMI)

It was found that cockerels, irrespective of heat / cold stressed and untreated / BR treated, showed higher MMI value when compared to G I (Table 2). The macrophages collected from G IV cockerels exhibited the maximum index value (10.56) followed by the G VI (5.61).

The peritoneal macrophages obtained from the cockerels of G IV and G VI showed significantly ($P<0.05$) greater area of migration when compared to their

Table 1. Plasma corticosterone level, after 5 days of heat / cold stress (n=8)

Groups	Plasma corticosterone (µg/ml)
G I	$41.00^{c} \pm 0.33$
G II	$31.00^{d} \pm 0.28$
G III	$82.50^{a} \pm 0.18$
G IV	$42.00^{b} \pm 0.20$
G V	$20.90^{e} \pm 0.32$
G VI	$20.40^{e} \pm 0.31$

One way Anova for comparing the variation between groups.

Mean ± SE having different superscripts (a-e) differ significantly ($P<0.05$) between groups.

G I – Non- heat / cold stressed and untreated control.

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

Table 2. Effect of heat / cold stress on peritoneal macrophage functions

(n = 8)

Groups	Phagocytosis (per cent)	L929 cytotoxicity (per cent)	Macrophage migration area (mm^2)	Macrophage migration index (MMI)
G I	$6.00^{d} \pm 0.57$	$70.50^{c} \pm 0.73$	$7.70^{e} \pm 0.48$	1.00
G II	$25.00^{b} \pm 0.87$	$84.50^{b} \pm 1.12$	$18.80^{d} \pm 1.36$	2.44
G III	$20.50^{c} \pm 0.68$	$58.50^{e} \pm 1.29$	$34.00^{c} \pm 3.87$	4.42
G IV	$30.75^{a} \pm 1.66$	$88.00^{a} \pm 1.01$	$81.30^{a} \pm 0.57$	10.56
G V	$19.00^{c} \pm 0.84$	$62.00^{d} \pm 0.57$	$33.90^{c} \pm 2.68$	4.40
G VI	$29.00^{a} \pm 1.35$	$70.00^{c} \pm 0.57$	$43.20^{b} \pm 2.64$	5.61

One way Anova for comparing the variation between groups.

Mean ± SE having different superscripts (a-e) differ significantly ($P<0.05$) between group

G I – Non- heat / cold stressed and untreated control .

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

MMI was derived by dividing the migration area of macrophages of each group (G II to G VI) with that of G I.

Plate 1.

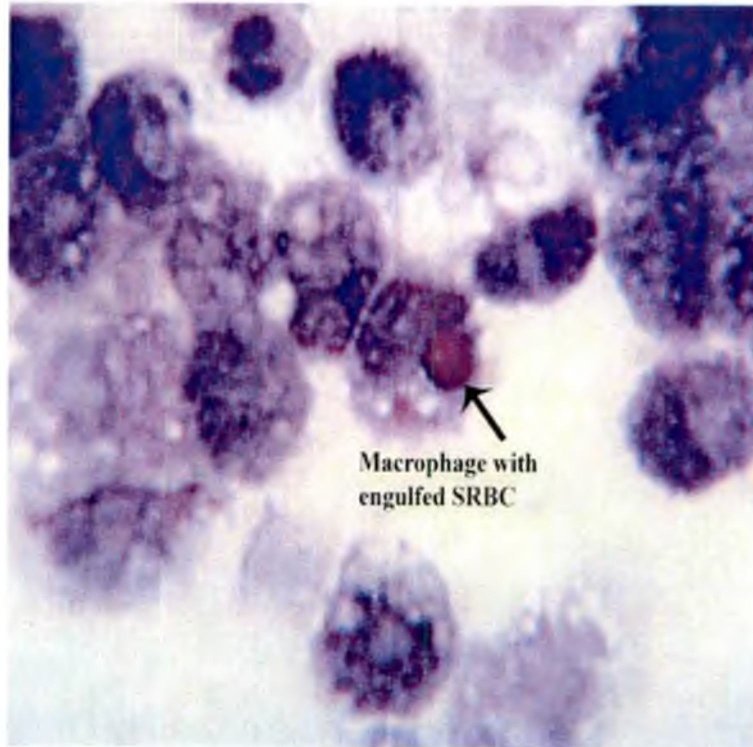


Fig. (i)

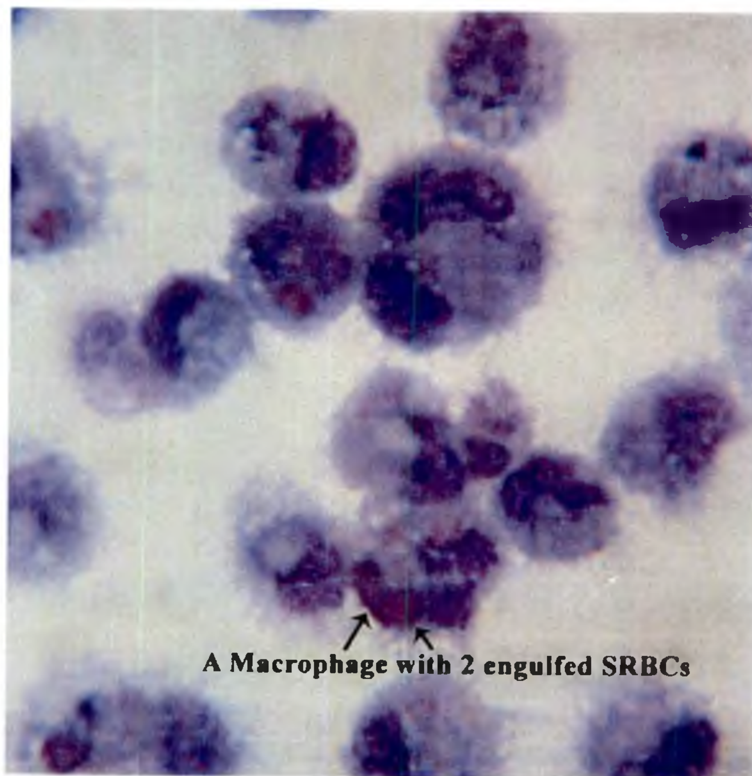


Fig. (ii)

Figures show macrophages (Giemsa stained 100X) with engulfed SRBC/s.

untreated counterparts. Peritoneal macrophages of G II showed significantly higher ($P<0.05$) migration area of 18.80 ± 1.36 mm^2 when compared to G I (Table 2).

4.4 EFFECT OF HEAT / COLD STRESS ON PHYTOHEMAGGLUTININ (PHA) ELICITED CUTANEOUS DELAYED HYPERSENSITIVITY RESPONSE

The mean toe web thickness showed no significant difference between groups at 0 h and 72 h post PHA inoculation in cockerels. But after 24 h of PHA inoculation, the toe web thickness was seen significantly ($P<0.05$) lowered in G V cockerels with 2.01 ± 0.14 mm, while the G II cockerels showed a maximum inflammatory thickness of 3.24 ± 0.20 mm. The mean toe web thickness of cockerels of G III was 1.90 ± 0.26 mm after 48 h of PHA inoculation and it was significantly lower ($P<0.05$) when compared to 2.71 ± 0.23 mm, shown by G IV cockerels (Table 3).

The mean toe web thickness between different time periods within group varied significantly ($P<0.05$) in all the groups except in G V cockerels. Cockerels of G I and G IV exhibited significant ($P<0.05$) increase in thickness upto 48 h of PHA inoculation. Cockerels of G II, G III and G VI showed a significant increase ($P<0.05$) only upto 24 h of PHA inoculation, which later on decreased to near normal values (Table 3).

4.5 EFFECT OF HEAT / COLD STRESS ON LEUCOCYTE MIGRATION INHIBITION INDEX (LMII)

Leucocytes separated from cockerels of G II underwent the maximum area of migration in bovine serum albumin (BSA) free wells (4.91 cm^2), followed by G VI (3.47 cm^2). The LMII was calculated by dividing the difference in the area of migration of WBCs in BSA free wells and BSA incorporated wells by that of BSA free wells. Inhibitory influences on leucocyte migration was significantly ($P<0.05$) noticed in G II, G IV, G V and G VI in decreasing order. Heat stressed cockerels of G III exhibited the minimum LMII of 0.206 ± 0.030 when compared to other groups.

Table 3. Effect of heat / cold stress on phytohemagglutinin (PHA) elicited cutaneous delayed hypersensitivity response (n=8)

Groups	Toe web thickness in mm			
	0 h	24 h	48 h	72h
G I	$_{a}1.71^{B} \pm 0.19$	$_{a}2.73^{A} \pm 0.17$	$_{ab}2.31^{A} \pm 0.19$	$_{a}2.17^{AB} \pm 0.19$
G II	$_{a}1.70^{C} \pm 0.18$	$_{a}3.24^{A} \pm 0.20$	$_{ab}2.51^{B} \pm 0.20$	$_{a}2.06^{BC} \pm 0.21$
G III	$_{a}1.69^{B} \pm 0.26$	$_{a}2.77^{A} \pm 0.25$	$_{b}1.90^{B} \pm 0.26$	$_{a}1.91^{B} \pm 0.15$
G IV	$_{a}1.70^{B} \pm 0.16$	$_{a}2.97^{A} \pm 0.24$	$_{a}2.71^{A} \pm 0.23$	$_{a}1.71^{B} \pm 0.17$
G V	$_{a}1.69^{A} \pm 0.14$	$_{b}2.01^{A} \pm 0.14$	$_{ab}2.03^{A} \pm 0.17$	$_{a}1.77^{A} \pm 0.21$
G VI	$_{a}1.71^{B} \pm 0.17$	$_{a}3.03^{A} \pm 0.17$	$_{ab}2.13^{B} \pm 0.17$	$_{a}1.99^{B} \pm 0.28$

One way Anova for comparing the variation between groups and split on time analysis for comparing the variation within group at different periods.

Mean ± SE bearing different superscripts (A-C) differ significantly ($P<0.05$) between different periods within group.

Mean ± SE bearing different subscripts (a-b) differ significantly ($P<0.05$) between groups.

G I – Non- heat / cold stressed and untreated control .

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

Maximum inhibition was characterized by minimum migration and it was encountered in G II cockerels (0.715 ± 0.003) [Table 4 and Plate 2 (Fig i)].

4.6 EFFECT OF HEAT / COLD STRESS ON SPLENOCYTE PROLIFERATION ASSAY

Results indicated that cockerels of G II, G IV, G V and G VI exhibited higher values, indicating significantly ($P<0.05$) higher rate of *in vitro* proliferation of splenocytes in mitogen free culture. When splenocytes were cultured in the presence of PHA at 2.5 μg as well as 5 μg per $5x10^4$ cells, significantly ($P<0.05$) increased proliferative response with a decay value of 5102 ± 27 cpm and 5545 ± 23 cpm respectively was noticed in G IV cockerels, when compared to other groups. It was also found that splenocytes, irrespective of different groups, proliferated to a greater extend in the culture containing 5 μg PHA per $5x10^4$ splenocytes when compared to the lower concentration of PHA (2.5 μg) (Table 5).

The proliferative response induced by Con A on splenocytes at the level of 5 μg in the culture brought about significantly ($P<0.05$) higher decay value of 6875 ± 29 cpm in G IV cockerels, while Con A at 10 μg incorporation level resulted in a decay level of only 5459 ± 27 cpm (Table 5).

4.7 EFFECT OF HEAT / COLD STRESS ON NATURAL KILLER (NK) CELL MEDIATED CYTOTOXICITY ASSAY

The ^{51}Cr labeled K562 cells which underwent lysis through NK cell interaction, discharged the radioisotope into the supernatant and its radioactivity was measured in triplicates (Table 6). Irrespective of heat / cold stressed or unstressed cockerels, administration of BR resulted in an increased activity of NK cells of spleen against K562 cells. However, the maximum NK cell cytotoxicity in terms of per cent K562 cell lysis in G II, G IV, and G VI cockerels were achieved on day 11 of the experiment as 83.5 ± 0.18 per cent, 94.0 ± 0.15 per cent and 95.0 ± 0.11 per cent respectively. Corresponding higher values of lysis were obtained on day 15 in G III

Table 4. Effect of heat / cold stress on leucocyte migration inhibition index (LMII)

(n = 8)

Groups	Mean WBC migration area in BSA wells (cm^2)	Mean WBC migration area in BSA free wells (cm^2)	LMII
G I	2.04	3.42	$0.403^{c} \pm 0.008$
G II	1.40	4.91	$0.715^{a} \pm 0.003$
G III	2.04	2.57	$0.206^{d} \pm 0.030$
G IV	0.82	2.67	$0.693^{a} \pm 0.020$
G V	0.28	0.65	$0.569^{b} \pm 0.010$
G VI	1.57	3.47	$0.548^{b} \pm 0.020$

One way Anova for comparing the variation between groups.

Mean ± SE having different superscripts (a-d) differ significantly (P<0.05) between groups.

G I – Non- heat / cold stressed and untreated control .

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

$$\text{LMII} = \frac{\text{Area of migration of WBC in (BSA free wells - BSA incorporated wells).}}{\text{Area of migration of WBC in BSA free wells}}$$

BSA - Bovine serum albumin.

Plate 2.

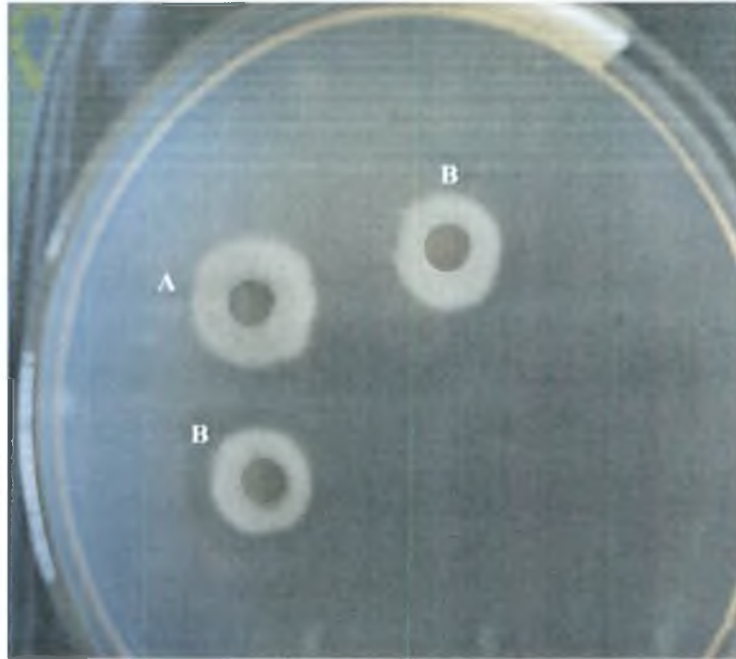


Fig. (i) Leukocyte migration inhibition test (LMIT).

A. represents antigen (BSA) free well with WBCs.
B. represent wells with both antigen and WBCs.

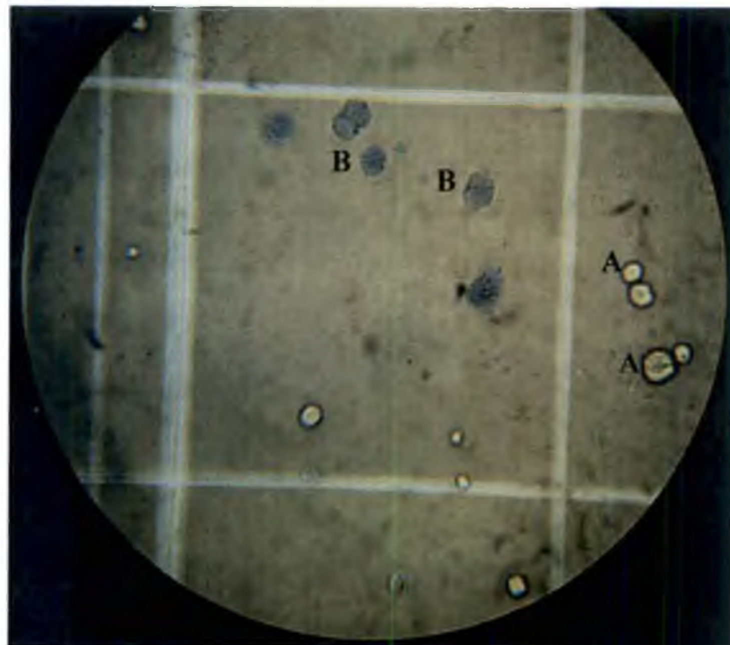


Fig. (ii) Antibody dependent complement mediated cytotoxicity (ACC) assay.

A. represent unstained live cells (40X).
B. represent dead EAC cells stained with trypan blue (40X).

Table 5. Effect of heat / cold stress on splenocytes proliferation assay (Mean ± SD values show decay of ^{3}H in cpm)

Groups	Without mitogen	PHA 2.5µg	PHA 5 µg	Con A 5 µg	Con A 10 µg
G I	$3426^{c} \pm 31$	$4876^{c} \pm 33$	$5088^{c} \pm 50$	$4842^{d} \pm 26$	$4444^{d} \pm 49$
G II	$4488^{b} \pm 18$	$5034^{b} \pm 21$	$5367^{b} \pm 31$	$5407^{c} \pm 20$	$4766^{c} \pm 19$
G III	$3414^{e} \pm 28$	$4010^{d} \pm 31$	$4223^{f} \pm 45$	$4810^{e} \pm 32$	$3913^{f} \pm 22$
G IV	$3936^{c} \pm 25$	$5102^{a} \pm 27$	$5545^{a} \pm 23$	$6875^{a} \pm 29$	$5459^{a} \pm 27$
G V	$3637^{d} \pm 39$	$4537^{d} \pm 24$	$4600^{e} \pm 18$	$4767^{f} \pm 45$	$4398^{e} \pm 22$
G VI	$4708^{a} \pm 21$	$4889^{c} \pm 31$	$4959^{d} \pm 31$	$5523^{b} \pm 30$	$5148^{b} \pm 23$

One way Anova for comparing the variation between groups.

Mean ± SD having different superscripts (a-f) differ significantly ($P<0.05$) between groups.

G I – Non- heat / cold stressed and untreated control .

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

cpm - counts per minute.

Table 6. Effect of heat /cold stress on natural killer (NK) cell activity

(Mean ± SD values show decay of ^{51}Cr in cpm)

Days	5	7	9	11	13	15	17	19
Total release	1064 ± 7	1062 ± 7	1060 ± 7	1391 ± 11	1292 ± 8	1240 ± 8	1189 ± 9	1136 ± 7
Spontaneous release	83 ± 1	120 ± 2	157 ± 2	120 ± 2	141 ± 2	120 ± 3	100 ± 2	86 ± 6
G I	100 ± 2	214 ± 3	337 ± 4	1191 ± 8	956 ± 4	1027 ± 7	972 ± 6	910 ± 6
G II	101 ± 2	205 ± 3	309 ± 4	1019 ± 7	844 ± 5	977 ± 7	972 ± 6	1073 ± 7
G III	101 ± 3	223 ± 3	243 ± 3	288 ± 3	247 ± 3	316 ± 4	258 ± 3	340 ± 5
G IV	102 ± 3	228 ± 4	345 ± 4	1217 ± 7	1014 ± 7	1128 ± 8	1107 ± 7	1073 ± 7
G V	98 ± 2	195 ± 4	297 ± 4	1013 ± 7	836 ± 5	960 ± 7	957 ± 6	931 ± 6
G VI	100 ± 2	233 ± 4	310 ± 4	1224 ± 8	1020 ± 7	1139 ± 8	1114 ± 9	1083 ± 7

G I – Non- heat/cold stressed and untreated control .

G II – Non- heat/cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

cpm - counts per minute.

cockerels (22.77 ± 0.14 per cent) and on day 17 in G V cockerels (80.0 ± 0.15 per cent) (Table 7 and Fig 1).

4.8 EFFECT OF HEAT / COLD STRESS ON ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY (ADCC) RESPONSE

The ^{51}Cr labeled SRBCs which when underwent lysis through ADCC, discharged the radioisotope into the supernatant and the radioactivity measured in triplicates (Table 8). The maximum ADCC activity in all the groups irrespective of heat / cold stressed or BR treated / untreated, expressed in terms of per cent lysis was obtained on day 9 of the experiment. It was found that cockerels in G IV exhibited the highest per cent lysis of SRBCs through ADCC (80 ± 0.15 per cent) when compared to all other groups, while G III cockerels showed the least peak of ADCC activity (27.8 ± 0.11 per cent). The peak ADCC activity was shown to be enhanced in cockerels of G II (68.51 ± 0.12 per cent) and G VI (60.0 ± 0.18 per cent) when compared to their untreated counterparts (Table 9 and Fig 2).

4.9 EFFECT OF HEAT / COLD STRESS ON ANTIBODY DEPENDENT COMPLEMENT MEDIATED CYTOTOXICITY (ACC)

In the present study it was found that anti-EAC containing serum, which with the help of complement induced a significantly higher ($P<0.05$) percentage of cell death on 5th day through ACC in all groups of cockerels when compared to their corresponding values on 10th day.

Anti-EAC serum obtained from cockerels of G IV and G VI brought about significantly ($P<0.05$) higher incidences of EAC cell death through ACC when compared to their untreated counterparts, on both the days tested. Percentage cell death of EAC was as high as 77.88 ± 0.60 (on 5th day) and 72.00 ± 0.33 (on 10th day) in G IV cockerels, while lower incidence of cell death was noticed in cockerels of G III with 37.66 ± 0.33 per cent (on 5th day) and 29.00 ± 0.40 per cent (on 10th day) [Table 10, Fig 3 and Plate 2 (Fig ii)].

Fig 1. Effect of heat / cold stress on natural killer (NK) cell activity against K562 cells

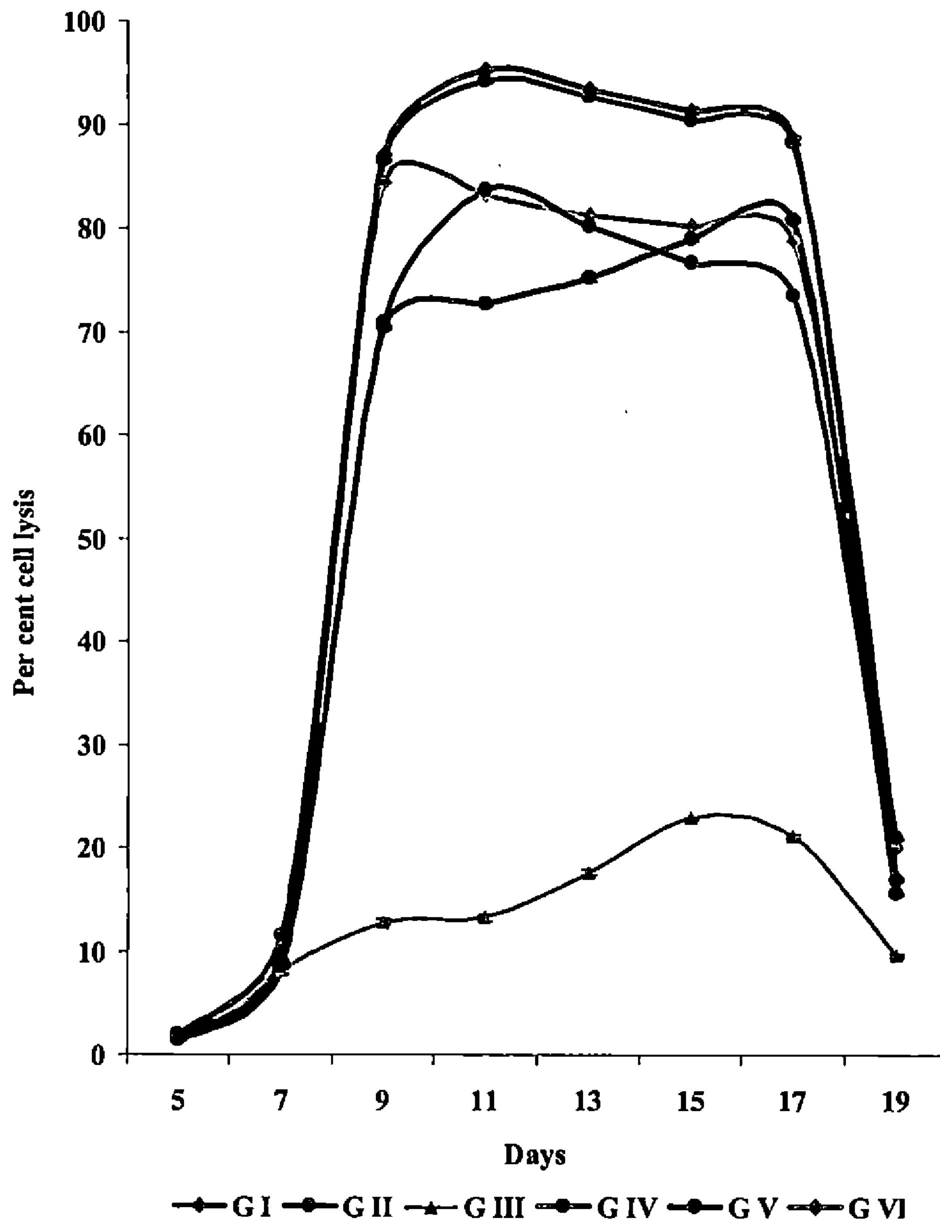


Table 8. Effect of heat / cold stress on antibody dependent cellular cytotoxicity (ADCC) response (Mean ± SD values show decay of ^{51}Cr in cpm)

Days	5	7	9	11	13	15	17	19
Total release	59 ± 2	59 ± 2	58 ±11	47 ± 7	66 ±10	68 ± 9	71 ± 9	74 ±10
Spontaneous release	5 ±4	5 ±3	4 ±3	7 ±4	5 ±4	6 ±4	9 ±5	9 ±4
G I	16 ± 4	25 ± 6	29 ± 5	21 ± 6	32 ± 4	32 ± 5	36 ± 5	37 ± 6
G II	19 ± 7	32 ± 5	41 ± 8	26 ± 5	24 ± 4	28 ± 4	34 ± 5	34 ± 7
G III	18 ± 6	18 ± 8	19 ± 6	18 ± 7	19 ± 8	17 ± 3	18 ± 8	15 ± 6
G IV	25 ± 6	39 ± 5	43 ± 7	39 ± 5	38 ± 4	33 ± 5	25 ± 5	23 ± 4
G V	25 ± 4	9 ± 4	11 ± 4	23 ± 5	28 ± 7	28 ± 5	30 ± 8	29 ± 5
G VI	36 ± 5	24 ± 5	25 ± 5	31 ± 6	37 ± 7	43 ± 6	46 ± 9	47 ± 6

G I – Non- heat / cold stressed and untreated control .

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

cpm - counts per minute.

Table 9. Effect of heat / cold stress on antibody dependentcellular cytotoxicity (ADCC) mediated cell lysis of SRBCs

Days	Percentage of K562 cell lysis							
	5	7	9	11	13	15	17	19
G I	29.00 ± 0.15	37.50 ± 0.18	46.30 ± 0.15	44.26 ± 0.1	43.79 ± 0.1	43.55 ± 0.12	42.30 ± 0.11	35.00 ± 0.22
G II	34.50 ± 0.11	51.00 ± 0.29	68.51 ± 0.12	47.50 ± 0.1	40.32 ± 0.08	38.50 ± 0.09	35.20 ± 0.16	31.50 ± 0.13
G III	24.00 ± 0.07	26.00 ± 0.11	27.80 ± 0.11	27.40 ± 0.06	22.95 ± 0.08	18.20 ± 0.3	14.52 ± 0.08	9.50 ± 0.22
G IV	37.03 ±0.07	54.20 ± 0.07	80.00 ± 0.15	72.22 ± 0.09	61.80 ± 0.16	43.70 ± 0.2	25.81 ± 0.16	22.00 ± 0.37
G V	5.40 ± 0.08	9.00 ± 0.18	40.00 ± 0.29	37.70 ± 0.18	34.90 ± 0.2	33.87 ± 0.19	30.20 ± 0.07	12.96 ± 0.23
G VI	5.40 ± 0.16	22.00 ± 0.12	60.00 ± 0.18	59.80 ± 0.04	59.70 ± 0.18	59.68 ± 0.16	58.00 ± 0.18	38.89 ± 0.23

G I – Non- heat/cold stressed and untreated control .

G II – Non- heat/cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

$$\text{Per cent lysis} = \frac{\text{Experimental release- Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

Fig 2. Effect of heat / cold stress on antibody dependent cellular cytotoxicity (ADCC) against SRBCs

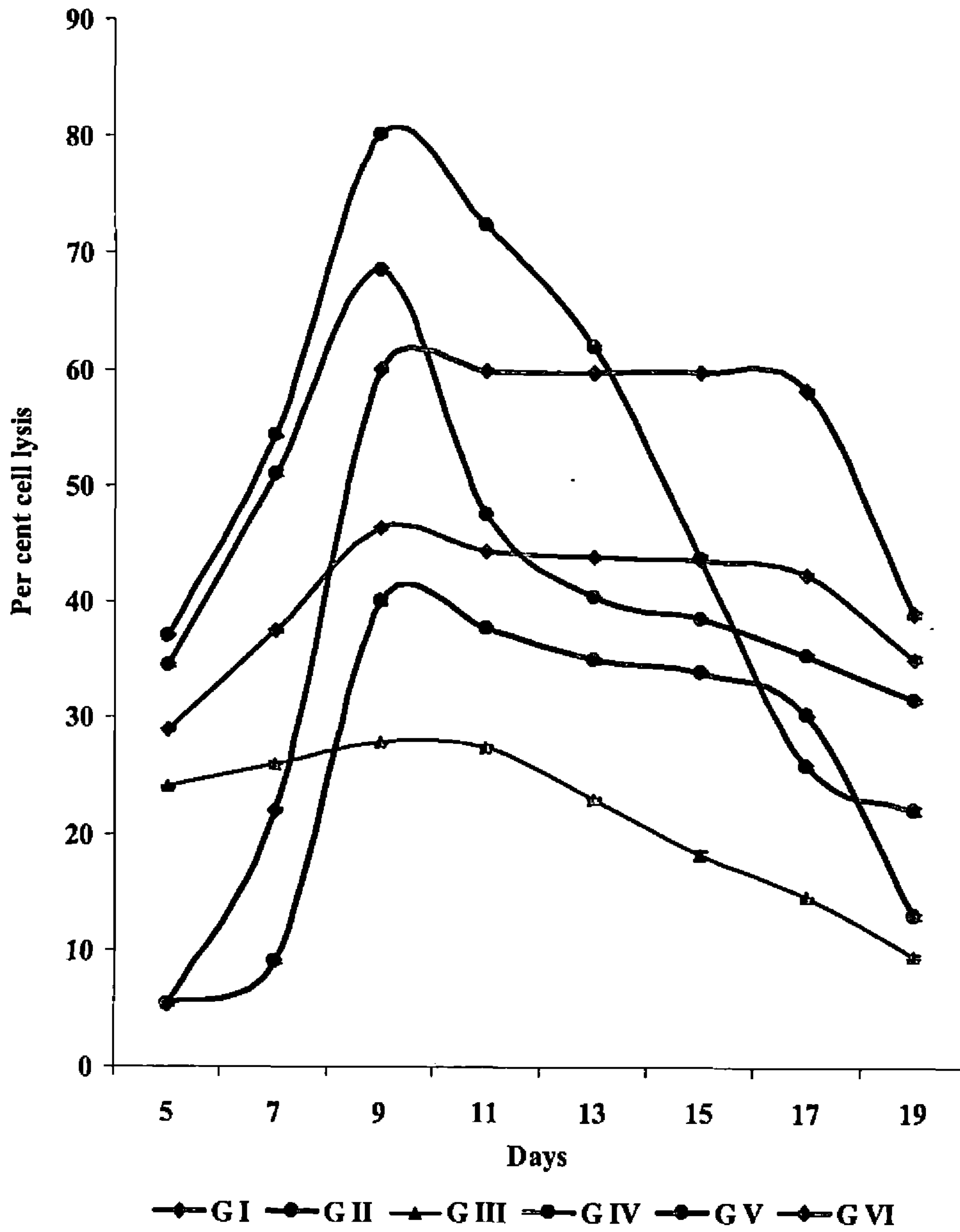


Table 10. Effect of heat / cold stress on antibody dependent complement mediated cytotoxicity (ACC) (n=8)

Groups	Percentage dead EAC cells	
	5th day	10th day
G I	$_{d}$50.00 ± 0.36	$_{c}$42.33* ± 0.35
G II	$_{c}$59.33 ± 0.35	$_{c}$42.33* ± 0.32
G III	37.66 ± 0.33	$_{e}$29.00* ± 0.40
G IV	$_{a}$77.88 ± 0.60	$_{a}$72.00* ± 0.33
G V	$_{e}$43.67 ± 0.28	$_{d}$40.00* ± 0.21
G VI	$_{b}$64.66 ± 0.38	48.00* ± 0.28

Student's t test for comparing variation between days 5 and 10 within group.

One way Anova for comparing the variation between groups.

Mean ± SE bearing * indicates significant difference between time periods at 5 per cent level, within group.

Mean ± SE having different subscripts (a-f), differ significantly ($P<0.05$) between groups.

G I – Non- heat / cold stressed and untreated control.

G II – Non- heat / cold stressed and BR treated (2 g/kg bw for 20 days, p.o.) control.

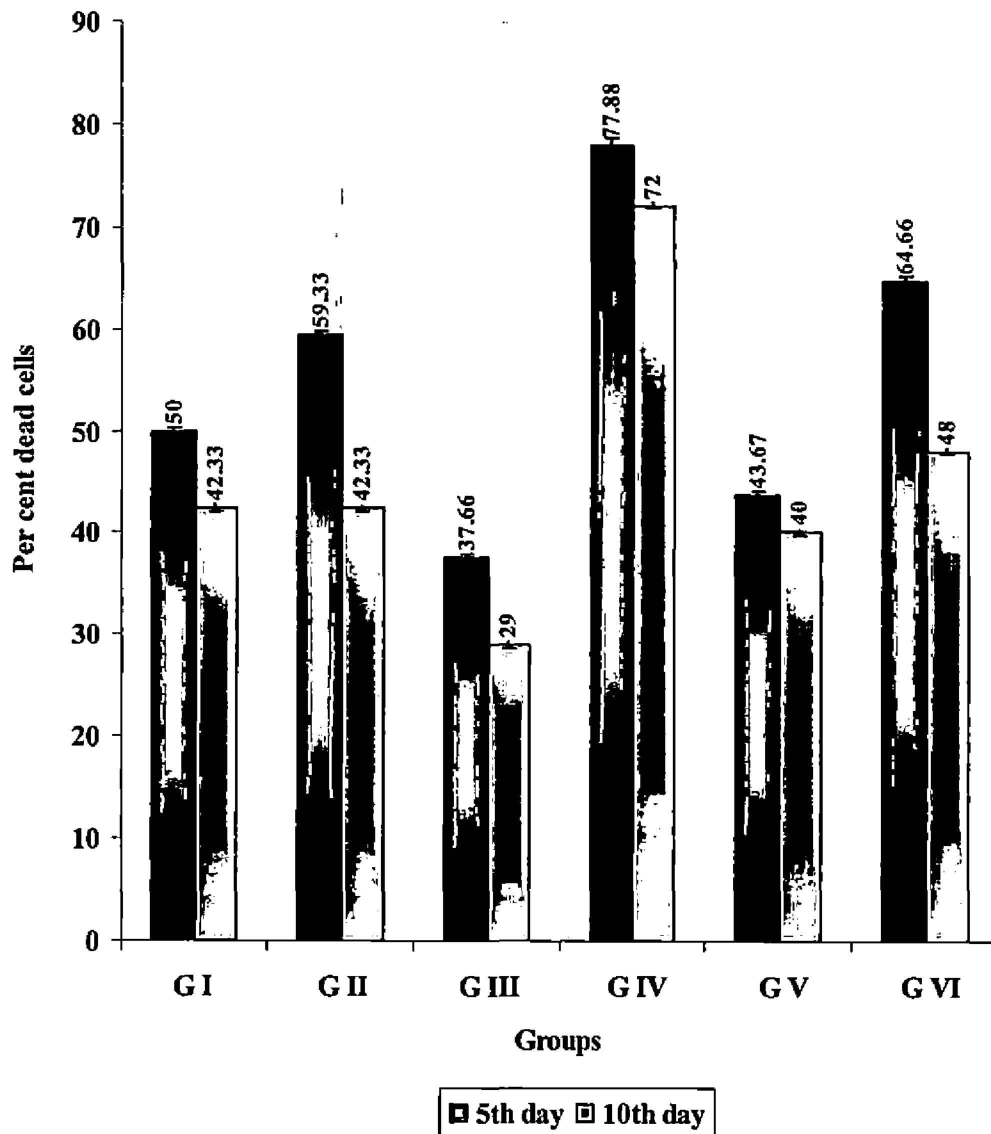
G III – HST and untreated.

G IV – HST and BR treated (10 days each prior to and during the heat stress).

G V – CST and untreated.

G VI – CST and BR treated (10 days each prior to and during the cold stress).

Fig 3. Effect of heat/cold stress on antibody dependent complement mediated cytotoxicity (ACC) against EAC cells



Discussion

5. DISCUSSION

Both innate and adaptive immunity depends upon the activity of leucocytes of blood. Innate immunity largely involves granulocytes and macrophages, whereas adaptive immune response depends upon lymphocytes, which provide the life long immunity that follows the exposure to disease causing organisms or vaccination.

Acquired immunity involves both humoral and cell mediated immune (CMI) reponses. Although both are effective against extracellular antigens, CMI responses are more specialized in the elimination of intracellular antigens and proteins of neoplastically transformed cells. The functional effectors of CMI responses are various immune cells like cytotoxic- T- lymphocytes, natural killer (NK) cells and macrophages.

Every animal experiences stress, which results in an oversecretion of glucocorticoids (Chrousos, 1995 and Karthiayini, 2007), which suppresses both innate and adaptive immune functions and make more susceptible to infections (Spehner *et al.*, 1996). Under farming systems birds are often exposed to various physical and climatic stressors (cold, heat, and wind), infectious diseases and social stress. Birds that are able to cope with or adapt to such adverse environmental conditions alone survive and perform.

5.1 EFFECT OF HEAT / COLD STRESS ON BODY TEMPERATURE

The present study revealed that the rectal temperature of both heat stressed and untreated / BR treated cockerels exhibited a rise of body temperature from 42.9 ± 0.2^0C to 44.0 ± 0.2^0C. This finding agreed with the reports of Yahav *et al.* (1997) who reported that exposure of chickens to high temperature induced hyperthermia. The temporary or passive hyperthermia could also be due to accelerated muscular work associated with increased respiratory and cardiovascular activity, as well as by increased metabolic heat production as reported by Siegel (1980).

Birds are non-sweating animals and they normally regulate the body temperature by vasomotor control of the surface blood supply, especially through featherless areas of the skin like comb and wattles as well as by vascular convection. Birds also depend on evaporatory heat loss, from respiratory system by polypnea, utilizing $^1/_3{}^{rd}$ of the normal tidal volume (Frankel *et al.*, 1962). The breed Gramapriya being evolved from White Leghorns are able to withstand higher temperature. The White Leghorns have been proved to withstand high environmental temperature because of their willingness to drink large amounts of water which improves evaporative cooling mechanism of the body (Ota and McNally, 1961).

The cockerels exposed to heat, lost an average 3.4 per cent bw and this findings agreed with reports of McKee *et al.* (1997) who stated that in hot environment, the rate of water loss (evaporative and excretory) exceeded the production of metabolic water leading to loss of body weight and survival thereafter depended on the availability of water.

The present study also revealed that the rectal temperature of cockerels exposed to cold stress for 4 h consecutively per day, resulted in a narrow reduction in body temperature from 42.9 ± 0.2^0C to 42.0 ± 0.2^0C. This finding agreed with Luger *et al.* (2003) who reported that acute cold stress induced hypothermia in birds. Chickens adapt to cold stress by reallocation of resources from body weight gain to thermoregulation and immune parameters (Hangalapura *et al.*, 2004). The results of present study closely agreed with an earlier observation made by Dabbert *et al.* (1997) who also found that exposure of birds to extreme cold would elevate existing metabolism by three-fold over basal metabolic rate, and the loss of body mass in cold stressed birds indicated that fat reserves were partly used to fuel these metabolic demands, despite the availability of feed.

5.2 EFFECT OF HEAT / COLD STRESS ON PLASMA CORTICOSTERONE LEVEL

Corticotropin releasing factor is released from the median eminence of the hypothalamus in response to stress, which stimulates the synthesis of ACTH (Krieger, 1983). Corticosterone is the major stress steroid in poultry and rats. The findings of present study revealed a significant increase ($P<0.05$) in plasma corticosterone level in heat stressed and untreated cockerels, when compared to non-stressed and untreated ones. This finding agreed with reports of Lin *et al.* (2006) who had noticed an increase in corticosterone level in acute heat stressed broiler chicks. Edens (1978) reported a sharp increase followed by a rapid decline in plasma corticosterone in heat stressed broilers.

Results of the present study indicated that circulating levels of plasma corticosterone gets lowered in BR treated cockerels and cold stressed cockerels. This finding was in close agreement with Djordjevic *et al.* (2003) who reported cold stress was less potent in elevating plasma ACTH levels. The present study also revealed a significant ($P<0.05$) decrease in plasma corticosterone level in cold stressed and untreated / BR treated cockerels when compared with non- heat / cold stressed and untreated cockerels. These findings matched with the reports of earlier workers (Shinder *et al.*, 2002 and Luger *et al.*, 2003), who reported a significantly lower level in plasma glucocorticoids on chronic cold exposure in broiler chickens.

De Nicola *et al.* (1968) reported that ACTH produced during stress inhibited ascorbic acid (AA) transport into the rat adrenals, resulting in significantly increased steroidogenesis and increased elimination of ascorbate. Numerous studies suggested that AA functioned as a regulator of adrenal steroids because high levels of AA had been found to inhibit steroidogenesis (Kitabchi, 1967), by inhibiting the enzymatic side chain cleavage system that converts cholesterol to pregnenolone (Shimizu, 1970). Ascorbic acid has also been shown to inhibit C-21 hydroxylase and 11-β hydroxylase in steroidogenic pathways (Cooper and Rosenthal, 1962) and thereby,

limiting some of the deleterious responses associated with stress. Gooseberry is one of the major ingredients of BR, being considered as a robust source of AA / Vit. C among the available herbs worldwide (Khandelwal *et al.*, 2002). Hence the amelioration of heat induced effects noticed in this study might be attributed to properties of BR by reducing glucocorticoids output and thereby protecting cells from the cytotoxic effects of adrenal steroids. This finding also coincided with reports of Srikumar *et al.* (2005) who explained the treatment with Triphala resulted in a decreased corticosterone level in rats. It could be assumed that BR supplemented heat stressed birds could have attained enough AA in the adrenals during the period of experiment and thereby reduced the synthesis of corticosterone.

5.3 EFFECT OF HEAT / COLD STRESS ON CELL MEDIATED IMMUNE (CMI) RESPONSES THROUGH MACROPHAGES

Macrophages perform a wide variety of functions, including engulfment (phagocytosis) of foreign particles, destruction of bacterial and tumor cells (Qureshi *et al.*, 1986; Qureshi and Miller, 1991), and secretion of prostaglandins (Bonney and Davies, 1984) and cytokines that regulate activity of lymphocytes and other macrophages (Kimball, 1990).

5.3.1 Phagocytic activity of macrophages

The ability of macrophages to phagocytose and digest microbes is crucial to the host defense against microbial challenge. For this reason the macrophage is considered as the first line of defense against microbial infection (Morgensen, 1979; Abbas *et al.*, 2000). In the present study peritoneal macrophages isolated from heat / cold stressed and BR treated cockerels showed significantly ($P<0.05$) higher phagocytic activity than non-stressed and untreated cockerels, against opsonised SRBCs. Chicken macrophages have been found to express surface receptors for the Fc (Duncan and McArthur, 1978) and for C3b (Dietert *et al.*, 1991), which enhance their ability to engulf opsonised particles. Like mammalian macrophages, avian

macrophages are capable of engulfing both opsonised and unopsonised particles, with opsonised particles being more efficiently phagocytized (Qureshi *et al.*, 1986).

It was reported that macrophage cultures when exposed to heat-shock conditions were able to maintain their Fc-mediated phagocytosis of antibody-coated SRBCs due to the reduced cell membrane turnover of surface proteins, thereby maintaining a higher incidence of Fc-receptors on the outer membrane (Miller and Qureshi, 1992b). This could be attributed as one of the reasons encountered for higher phagocytic activity of macrophages in heat / cold stressed cockerels in this study. The findings of Hangalapura (2004) that cold stress of 10^0C stimulated phagocytosis, also supported the results of present trial. Dietary supplementation with natural compounds such as *Spirulina platensis* (blue-green algae) had been shown to enhance the clearance of *Escherichia coli* and *Staphylococcus aureus* from systemic circulation and spleen, by activating mononuclear phagocytic system of chickens (Qureshi *et al.*, 1995). In the present study, the dietary supplementation of BR had proved to be beneficial as during heat / cold stress, treated cockerels exhibited significantly ($P<0.05$) higher phagocytic potential against opsonised SRBCs than their untreated counterparts.

5.3.2 Cytotoxic activity of macrophages

As the first line of immunological defense, monocytes / macrophages are obvious targets for various environmental stressors and toxins. It has been found that macrophages with phagocytized bacteria, on receiving IFN-γ from Th1 effector cells, increased the production of reactive oxygen intermediates, oxygen-independent microbial factors (transferrin and cationic proteins), and inflammatory cytokines such as tumor necrosis factor-α, IL-1, IL-6, and IL-8 that stimulated inflammation, leucocyte recruitment and granuloma formation, in order to contain and eliminate the infection (Erf, 2004). Certain dietary materials induce macrophage's antimicrobial effects by way of increasing the nitric oxide synthase gene expression, leading to enhanced arginine metabolism and nitric oxide production against pathogenic

intracellular bacteria, viruses and protozoa (Al- Batshan *et al.*, 2001). The results of the present study indicated a lower percentage of L929 cytotoxicity produced by macrophages in heat / cold stressed and untreated cockerels. But the dietary supplementation of BR had proved to be beneficial as during heat / cold stress, BR treated cockerels exhibited significantly ($P<0.05$) higher tumoricidal activity.

5.3.3 Macrophage migration index (MMI)

Migration index of macrophages is an indicator of sensing chemotactic factors. It has been reported that chicken macrophages have cell surface markers namely, Mac-I and LFA, which are necessary for macrophage adherence and chemotaxis (Qureshi, 1998). The results of the present study indicated that irrespective of heat / cold stressed and untreated / BR treated, cockerels of all groups except non-stressed and untreated group showed significantly ($P<0.05$) higher migratory areas in the wells and corresponding index values of migration. Macrophages undergo some molecular and functional changes when exposed to heat stress both *in vivo* and *in vitro*. These changes are documented in terms of increased expression of heat-shock gene families. The effector functions of macrophages, especially migration potential, have been positively correlated with expression of HSPs. Synthesis of HSPs was often increased even in non-thermal stress. The HSPs seem to maintain integrity and other functions of cells during environmental stress (Miller and Qureshi, 1992a). So it can be assumed that a heat / cold stress would have increased the synthesis of HSPs which might have increased the migratory capability of macrophages of heat / cold stressed and untreated / BR treated cockerels than the controls.

The migration of peritoneal macrophages is markedly inhibited in most animals and birds only when there is interference by antigen sensitized peripheral blood lymphocytes. The sensitized lymphocytes are able to release, upon interaction with antigen, various soluble factors sufficient to induce the inhibition of macrophage migration (Ricci *et al.*, 1969). The above finding support the outcome of the present

trial as the migratory potential of peritoneal macrophages during heat / cold stress which was seen to be increased could be attributed to the non interference of T / B lymphocytes.

The oral administration of BR at the rate of 2 g/kg bw had again proved in this study, to be a modulator of macrophage function with respect to its migratory potential, especially during heat / cold stress.

5.4 EFFECT OF HEAT / COLD STRESS ON DELAYED TYPE HYPERSENSITIVITY (DTH) RESPONSE

Certain antigens when injected into the skin of sensitized animals provoke slowly developing inflammation at the injection site. Since this "delayed" hypersensitivity reaction can only be transferred from sensitized to normal animals by T lymphocytes, it must be cell mediated one. The DTH results from interactions among the injected antigen, antigen presenting cells, and T lymphocytes. The Langerhans cells which are specialized dendritic cells present in the skin possess high levels of surface major histocompatibility complex (MHC) class II molecules and process and the present peptide antigens to T lymphocytes (Tizzard, 2004).

Cutaneous basophil hypersensitivity elicited in chickens by an intradermal injection of PHA-P is a thymus dependent response mediated by T lymphocytes (Brake *et al.*, 1988 and Corrier and DeLoach, 1990). The acute response probably consists of local increased vasopermeability (oedema) due to the release of vasoactive amines such as serotonin or histamine by mast cells (Ptak *et al.*, 1991). In chicken, the development of antigen specific DTH occur immediately within 1-2 h post challenge, which is serotonin dependent. The mitogens are known to stimulate degranulation of mast cells and basophils as well as activate macrophages which release IL-1 and TNF α, all of which lead to local expression of adhessines, followed by extravascularisation of leucocytes. This leads to local homing of lymphocytes towards the sensitizing agent and production of DTH reactions on the skin (Parmentier *et al.*, 1998)

The results of the present study had shown that a significantly ($P<0.05$) decreased delayed hypersensitivity response in heat as well as cold stressed untreated cockerels at 24 as well as 48 h post intradermal PHA challenge, when compared to their BR treated counterparts. These results partially agreed with Regnier and Kelley (1981) who reported that the exposure to heat and cold stress reduced *in vitro* and *in vivo* CMI response to PHA-P in chicks. Corticosterone is reported to possess suppressive effects on cutaneous basophil hypersensitivity response elicited by PHA (Brake *et al.*, 1988). The data obtained in this study regarding corticosterone level in plasma might be responsible for the reduced delayed hypersensitivity response in heat stressed untreated birds at 48 h post PHA challenge.

5.5 EFFECT OF HEAT / COLD STRESS ON LEUCOCYTE MIGRATION INHIBITION INDEX (LMII)

Leucocyte migration inhibition (LMI) test offers one of the simplest techniques for measuring cell mediated immune response. The LMI is widely used as *in vitro* correlate of DTH in mammals (Gilliland *et al.*, 1982). On the whole, 92 per cent of the migrating leucocytes are polymorphonuclear cells and 8 per cent lymphocytes / monocytes.

The results of the present study indicated that the cutaneous delayed hypersensitivity response at 48 h and LMII had been well correlated in heat stressed BR treated as well as untreated birds. A similar kind of correlation was observed in non stressed BR treated birds at 24 h. This was in agreement with an early report of Erard, (1974) who had shown a positive correlation between DTH and LMII. Certain factors like neutrophil immobilization factor are released by polymorphonuclear leucocytes upon exposure to some of the soluble bacterial antigens (Erard, 1974) and endotoxins which directly and irreversibly inhibit the response of neutrophils to diverse chemotactic stimuli without impairing their viability (Senyk and Hadley, 1973; Erard, 1974 and Zimmermann and Felber, 1977). A high index of LMI was noticed in the study, especially in all the BR treated birds, irrespective of heat / cold

stress, which indicated the capacity of the drug to potentiate the CMI response of the subjects. As a result of inhibition of rapid migration through agarose, leucocytes got enough chance to process and eliminate the challenged antigens effectively.

5.6 EFFECT OF HEAT / COLD STRESS ON SPLENOCYTE PROLIFERATION ASSAY

Proliferation of lymphocytes and the subsequent release of cytokines are important events in any immune response. Lectins are considered to be lymphocyte stimulants that bind to sugar residues of cell surface glycoproteins and trigger cell division. The phytohemagglutinin (PHA; 26-30 KD) and concanavalin A (Con A; 106 KD) are isolated from plants. The PHA binds to N-acetyl galactosamine, and Con A to α-mannose and α-glucose (Tizzard, 2004). Upon stimulation by mitogens, T cells produce IL-2 which inturn stimulates T cell clonal proliferation or blastogenesis (Niwano *et al.*, 1990).

Results of the present study indicated that within groups, 5 μg of PHA when added to $5x10^4$ splenocytes, induced a greater response when compared to 2.5 μg level inclusion. This dose dependent proliferative response was dampened in heat / cold stressed untreated cockerels. However, by BR supplementation, proliferative response of splenocytes in heat / cold stressed cockerels could be enhanced to near normal values. Similar findings are reported by Jacob, 1995; Retsky and Frei, 1995 and Rekha *et al.*, 2003.

It has been reported that the disruption of IL-2 production at the level of mRNA transcription as in case with dexamethazone treatment, resulted in the cessation of blastogenic response induced by mitogens (Oppenheim *et al.*, 1987). High circulating levels of corticosterone encountered in the present study, especially in the heat stressed and untreated cockerels, might be one of the attributable reasons that had diminished the T lymphocyte response to PHA. Unlike PHA, as far as blastogenesis response to Con A was concerned, the splenocytes ($5x10^4$) incubated with 10 μg Con A did not show any dose dependent response. Incubating Con A

(5 μg) with splenocytes had resulted significantly higher uptake of ^{3}H-thymidin in heat / cold stressed BR treated cockerels when compared to all other counterparts, suggesting that BR supplementation was beneficial, especially to immunosuppressed birds.

5.7 EFFECT OF HEAT / COLD STRESS ON NATURAL KILLER (NK) CELL MEDIATED CYTOTOXICITY AASAY

Natural killer cells are morphologically a subpopulation of large granular lymphocytes that are found in normal, unsensitized individuals and can recognize and kill abnormal cells such as tumor and virus-infected cells without prior sensitization *in vivo*. They are considered as the first level defense against spread of tumor (Barlozzari *et al.*, 1985 and Andreesen *et al.*, 1990).

Immune cells execute many of their functions through the production of various cytokines. Cytotoxic T lymphocytes (CTL) and NK activity play important roles in immunological surveillance in neoplasia and metastasis (Cooper *et al.*, 2001). In contrast to CTL, triggered by class I MHC molecules, NK cells mediate lysis of target cells that fail to express MHC class I molecules (Moretta *et al.*, 2002). The IL-2 stimulates NK cell and T cell proliferation (Misawa *et al.*, 2000), while IFN-γ, a key cytokine secreted by activated NK cells, interferes with the recognition and killing of target cells by upregulating class I MHC molecules and also exerts direct antitumor activity (Wodnar-Filipowicz and Kalberer, 2006).

Once triggered, NK cell killing is mediated, like T cells, through perforins and granzymes as well as through the death receptor pathway. Perforins and granzymes expressions are increased by exposure to IL-2 and IL-12. It produces characteristic small lesions in target cell surfaces. The NK cell also secretes a protease called fragmentin that can induce DNA fragmentation and apoptosis in target cells. The NK cells are thus thought to be a key factor in tumor rejection *in vivo* (Ehrhardt *et al.*, 1997).

The results of the present study indicated a peak of NK cell activity on 15th day and 17th day of heat and cold exposure respectively. The chicken, which had been subjected to heat / cold stress and BR treatment, attained an early peak of target cell lysis by 11th day. This was true for the non-stressed BR treated cockerels also.

The expression of NK cell activity at an early stage of the tumor mass development could be attained by enhancing the immunological status of the body. The data obtained in the present investigation indicated that BR supplementation could augment the cellular arm of immune response mediated through NK cells and this property could be advantageously used to alleviate the immunosuppressive effects of stress. Stressors that might inhibit NK cell activities would promote tumor growth. Use of immunomodulating agents like BR can solve this problem largely.

5.8 EFFECT OF HEAT / COLD STRESS ON ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY (ADCC) RESPONSE

The ADCC represents a link between the humoral and cell mediated immunity. This involves the killing of antibody coated target cells by cytotoxic T lymphocytes with surface Fc receptors. The cells that possess the Fc receptors bind to foreign target cells or bacteria through specific antibodies and then become cytotoxic. Thus ADCC has been nominated as another mechanism of antitumor effects of lymphoid cell populations that are lacking surface immunoglobulin, but bear Fc receptors. These cytotoxic cells include K-cells, NK-cells, neutrophils and monocytes / macrophages involving polyclonal or monoclonal antibody directed lysis against the target cells (Goldsby *et al.*, 2002). However, the ADCC is slower and less efficient than direct T cell mediated cytotoxicity, taking from 6 to 18 h to occur (Tizzard, 2004).

In the present study, a peak ADCC activity as observed by the increased lysis of the target SRBC cells was exhibited on day 9th of the trial by cockerels of all groups, irrespective of temperature stress or BR treatment. Administration of BR could

increase the ADCC activity in heat / cold stressed and non-stressed cockerels, revealing its immunopotentiating property of in stress.

5.9 EFFECT OF HEAT / COLD STRESS ON ANTIBODY DEPENDENT COMPLEMENT MEDIATED CYTOTOXICITY (ACC) RESPONSE

Cell mediated immune response can also be assessed by complement mediated cytolysis. The complement system consists of about 30 serum proteins that are activated in response to various stimuli, known as the "complement cascade," ensuring the killing of altered cells (Goldsby *et al.*, 2002). The complement can be activated by antibody, leading to a cascade of reactions that occurs on the surface of pathogens and generates active components with various effector functions. Complement proteins are also responsible for cell lysis and mediation of inflammation and enhanced phagocytosis.

Antibodies kill infected cells / tumor cells by complement mediated cytolysis in which the cytotoxic cells such as lymphocytes, macrophages and neutrophils participate through their Fc receptors to bind to antibody coated target cells. An enhanced ACC activity as measured from the percentage cell death of EAC cells *in vitro*, was obtained on day 5th, when compared to day 10th, in all the groups of cockerels in this study. The ACC response was shown to be significantly ($P<0.05$) enhanced in BR treated cockerels. More than two fold increase in activity was observed in heat stressed and BR treated cockerels as compared with their untreated counterparts. A significant increase in ACC response was also shown by BR treated cold stressed cockerels and BR treated non-stressed cockerels, when compared to their untreated counterparts. These results provided an evidence for the immunomodulatory potency of the BR in heat / cold stressed chicken.

Studies involving the effect of various plant based biomaterials on the CMI responses evaluated through NK, ADCC and ACC assays in different species of animals were reported by Kumar *et al.*, 1999 using extracts of *Viscum album*, Kuttan and Kuttan, 1992 using extracts of *Withania somnifera*, Manu and Kuttan, 2007 using

punarnavine and Sheeja and Kuttan, 2007 with andrographolide. Most of the herbs screened were found to potentiate CMI responses similar to the one obtained in this study for the BR in heat / cold stressed cockerels.

These data thus indicated that BR could result in a significant stimulation of CMI responses and this property could be advantageously used to alleviate the immunosuppressive effects of stress. As a continuation of the present study further research has to be undertaken with respect to the role of heat shock proteins produced by leucocytes that take part in cell mediated immune responses in birds. Further investigations are also required to understand the role of various cytokines during temperature stress.

Summary

6. SUMMARY

From the results of the present investigation, it can be concluded that heat and cold stress resulted in a marked reduction in the cell mediated immune status of cockerels. Both heat and cold stress could suppress the cellular immune responses measured in terms of peritoneal macrophage functions, lymphocyte functional assays, cutaneous delayed hypersensitivity response and natural killer cell activities. In general, the severity of alterations on the above mentioned parameters could be reversed significantly by BR supplementation, which could be attributed to the immunopotentiating property of the drug.

The present study indicated that the exposure of cockerels to heat stress (40 ± 1^0C and RH 80 ± 5 per cent for 10 days @ 4 consecutive hours / day) resulted in a moderate hyperthermia, which could be attributed to higher metabolic heat production. On the other hand, as the consequence of cold stress (4 ± 1^0C and RH 45 per cent @ 4 consecutive hours / day) a decline in the body temperature of cockerels to 42 ± 1^0C was noticed in this study.

From the present study it was concluded that the heat stress could enhance the plasma corticosterone concentration to a greater extent whereas, the cold stress decreased the same. The supplementation of BR was shown to reduce the plasma levels of this hormone in cockerels under temperature stress.

The phagocytic potential of peritoneal macrophages isolated from cockerels exposed to both heat / cold stress against opsonised SRBCs showed a noticeable increase. This might be due to the stabilization of Fc-mediated receptors on cell membrane during heat shock conditions which are involved in the phagocytosis of antibody coated SRBCs. The BR supplementation could further enhance the phagocytic function.

The cytotoxicity of L929 cells by macrophages was significantly lowered in cockerels exposed to heat / cold stress. These changes could be described in terms of

increased expression of heat-shock gene families by macrophages and monocytes during temperature stress, which decrease the tumoricidal potential. However, the dietary supplementation of BR could augment the tumoricidal potential of macrophages against L929 cells.

The adherence followed by migration to wider areas by macrophages isolated from heat / cold stressed cockerels supplemented with BR, was found to be much higher than the untreated counterparts. This again revealed the immunopotentiating property of BR in stressed birds.

The results of the present study indicated that the cutaneous delayed hypersensitivity response measured by toe web thickness in heat as well as cold stressed untreated cockerels was reduced at 24 / 48 h post intradermal PHA inoculation, when compared to their BR treated counterparts.

Leucocytes isolated from non-stressed BR treated cockerels exhibited maximum inhibition for migration in the presence of antigen on agarose. Leucocytes of heat stressed untreated cockerels showed the least migration inhibitory capability in the presence of antigen, which indicated the leucocytes' lowered capacity to process the challenged antigen and eliminate the same. It could be attributed to the immunosuppressive effects of corticosterone in heat stressed untreated cockerels.

As far as splenocytes mitogen induced proliferative response was concerned, in the present study it was found that PHA could induce a dose dependent blastogenic response in all groups of cockerels. The Con A at a concentration of 5 µg per $5x10^4$ splenocytes induced much higher proliferation than at 10 µg of inclusion. The BR supplementation also resulted in greater proliferative response irrespective of temperature stress.

The NK cell activity was assessed from K562 cell lysis. The oral supplementation of BR to heat / cold stressed and non-stressed cockerels resulted in an early achievement of peak NK cell activity by 11th day of the stress period. The results of the present study indicated that the peak NK cell activity was obtained on

day 15 and day 17 of the heat and cold stress respectively. Results indicated that the BR alleviated the immunosuppressive effects of stress.

From the present study, it was found that the maximum antibody dependent cellular cytotoxicity measured from SRBC lysis was on 9th day in all groups of cockerels. However, the BR administered cockerels exhibited a higher peak of ADCC activity in both heat / cold stressed and unstressed conditions, suggesting the immunopotentiative property of the drug.

The antibody dependent complement mediated cytotoxicity response in the present study had shown a significant enhancement of EAC cell death in BR treated heat / cold stressed and non-stressed cockerels, when compared to their untreated counterparts. It was also noticed that the *in vitro* per cent of cell death of EAC cells was higher on day 5, when compared to day 10, in all the groups of cockerels. This could be due to the potentiative action of BR on CMI responses. It also confirmed BR's property to overcome the immunological alterations encountered during stress.

References

REFERENCES

Abbas, A. K., Lichtman, A. H. and Pober, J. S. 2000. *Cellular and molecular immunology*. Fourth edition. WB Saunders, Philadelphia

Abbas, A.O., Gehad, A.E., Hendricks III., Gharib, G.L. and Mashaly, M.M. 2007. The effect of lighting program and melatonin on the alleviation of the negative impact of heat stress on the immune response in broiler chickens. *Int. J. Poult. Sci.* 6(9): 651-660

Al- Batshan, H.A., Al- Mufarrej, S.I., Al- Homaidan, A.A. and Qureshi, M.A. 2001. Enhancement of chicken macrophage phagocytic function and nitrite production by dietary *Spirulina platensis*. *Immunopharmacol. Immunotoxicol.* 23(2): 281-289

Altan, O., Altan, A., Cabuk, M. and Bayraktar, H. 2000. Effects of heat stress on some blood parameters in broilers. *Turk. J. Vet Anim. Sci.* 24: 145-148

Andreesen, R., Scheibenbogen, C., Brugger, W., Krause, S., Meerpohl, H.G. and Lesser, H.G. 1990. Adoptive transfer of tumor cytotoxic macrophages generated *in vitro* from circulating blood monocytes: a new approach to cancer immunotherapy. *Cancer Res.* 50: 7450-7456

Anonymous, 1978. *The Ayurvedic Formulary of India*. Ministry of health and Family Planning, New Delhi

Barlozzari, T., Leonhardt, J., Wiltrout, R.H., Herberman, R.B. and Reynolds, C.W. 1985. Direct evidence for the role of LGL in the reduction of experimental tumor metastasis. *J. Immunol.* 134: 2783-2789

Belsheim, J. 1981. A modified leukocyte migration under agarose technique (LMAT). Technical aspects. *Acta Pathol. Microbiol. Scand.* 89(3): 167-174

Bendixen, P. H. 1977. Application of the direct leucocyte migration agarose test in cattle naturally infected with *M. paratbc. Am. J. Vet. Res.* 38: 66-70

Blanckmeister, C.A. and Susdorf, D.H. 1985. Macrophage activation by cross- linked dextran. *J.Leukoc. Biol.* 37(2): 209-219

Bonney, R.J. and Davies, P. 1984. Possible autoregulatory functions of the secretory products of mononuclear phagocytes. *Contemp. Top. Immunobiol.* 14: 199-223

Brake, N.P., Brake, J., Thaxton, J.P. and Murray, D.L. 1988. Effect of cortisol on cutaneous basophil hypersensitivity to phytohemagglutinin in chickens. *Poult. Sci.* 67: 669-673

Brown, K.I. and Nedtor, K.E. 1973. Some physiological responses of turkeys selected for high and low adrenal response to cold stress. *Poult. Sci.* 52: 1948-1954

Bureau of Indian Standards (BIS). 1992. *Poultry Feeds – specification.* Fourth revision. Bureau of Indian Standards, New Delhi. 4p

Campling, B.G., Phym, J., Barker, H.M., Cole, S.P and Lam, Y.M. 1991. Chemosensitivity testing of small cell lung cancer using MTT assay. *Br. J. Cancer.* 63: 75-83

Chhabra, P.C. and Goel, M.C. 1981. Immunological response of chickens to *Mycoplasma gallisepticum* infection. *Avian Dis.* 25(2): 279-293

Chi, D.S., Bhogal, B.S., Fox, G.J. and Thorbecke, G.J. 1984. Effect of temperature and lymphokines on mixed lymphocyte and mitogen responses of chicken lymphoid cells in vitro. *Dev. Comp. Immunol.* 8(3): 683-694

Chrousos, G.P., 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N. Engl. J. Med.* 332: 1351-1362

Cochran, F.R. and Baxter, C.S. 1984. Macrophage-mediated suppression of T lymphocyte proliferation induced by oral carrageenan administration. *Immunology*. 53(2): 291-297

Cole, S.P. 1986. Rapid chemosensitivity testing of human lung tumour cells using MTT assay. *Cancer Chemother. Pharmacol.* 17: 256-260

Cooper, D.Y. and Rosenthal, O. 1962. Action of noradrenaline and ascorbic acid in C-21 hydroxylation of steroids by adrenocortical microsomes. *Arch. Biochem. Biophysiol.* 96: 331-335

Cooper, M.A., Fehniger, T.A. and Caligiuri, M.A. 2001. The biology of human atural killer cell subsets. *Trend Immunol.* 22: 633–640

Corrier, D.E. and DeLoach, J.R. 1990. Evaluation of cell-mediated, cutaneous basophil hypersensitivity in young chickens by an interdigital skin test. *Poult. Sci.* 69: 403-408

Courreges, M.C., Benencia, F., Coto, C.E., Massoh, E.J. and Coulombie, F.C. 1994. *In vitro* antiphagocytic effect of *Melia azedarach* leaf extracts on mouse peritoneal exudate cells. *J. Ethnopharmacol.* 43(2): 135-140

Dabbert, C. B., Lochmiller, L.R. and Teeter, G.R. 1997. Effects of acute thermal stress on the immune system of the northern bobwhite (*Colinus virginianus*). *The Auk.* 114(1): 103-109

Davison, T.F. and Lickiss, P.A. 1979. The effect of cold stress on the fasted, water deprived, neonate chicken (*Gallus domesticus*). *J. Thermal Biol.* 4(2): 113-120

De Nicola, A.F., Clayman, M. and Johnstone, R.M. 1968. Hormonal control of ascorbic acid transport in rat adrenal glands. *Endocrinol.* 82: 436-446

Djordjevic, G.J., Cvijic, G. and Davidovic, V. 2003. Different activation of ACTH and corticosterone release in response to various stressors in rats. *Physiolo. Res.* 52: 67-72

Dietert, R.R., Golemboski, K.A., Bloom, S.E. and Qureshi, M.A. 1991. The avian macrophage in cellular immunity, in: J.M. Sharma (Ed.). Boca Raton, FL, CRC Press, Inc. *Avian Cellular Immunology*: 71-95

Duncan, R.L. and McArthur, W.P. 1978. Partial characterization and the distribution of chicken mononuclear cells bearing the Fc receptor. *J. Immunol.* 120: 1014-1020

Edens, F.W. 1978. Adrenal cortical insufficiency in young chickens exposed to a high ambient temperature. *Poult. Sci.* 57: 1746-1750

Ehrhardt, R.O., Ludviksson, B.R., Gray, B., Neurath, M. and Strober, W. 1997. Induction and prevention of chronic inflammation in IL-2 deficient mice. *J. Immunol.* 158: 566–573

Erard, P. 1974. Technical study of the leucocyte migration inhibition test in agarose. *Clin. Exp. Immunol.* 18: 439-448

Erf, G.F. 2004. Cell-mediated immunity in poultry. *Poult. Sci.* 83: 580-590

Frankel, H., Holland, K. and Weiss, H.S. 1962. Respiratory and circulatory response of hyperthermic chickens. *Arch. Int. Physiol. Biochem.* 70: 555-563

Fukui, H., Overton, W.R., Herberman, R.B. and Reynolds, C.W. 1987. Natural killer cell activity in the rat. VI. Characterization of rat large granular lymphocytes as effector cells in natural killer and antibody-dependent cellular cytotoxic activities. *J.Leukoc. Biol.* 41: 130-142

Fulzele, S.V., Satturwar, P.M., Joshi, S.B. and Dorle, A.K. 2003. Study of the immunomodulatory activity of *Haridradi ghrita* in rats. *Indian J. Pharmacol.* 35: 51-54

Gaddipati, J.P., Rajeshkumar, N.V., Thangapazham, R.L., Sharma, A., Warren, J., Mog, S.R., Singh, A.K. and Maheshwari, R.K. 2004. Protective effect of a

polyherbal preparation, brahma rasayana against tumor growth and lung metastasis in rat prostate model system. *J. Exp. Ther. Oncol.* 4(3): 203-212

Gandhi, N.M. and Nair, C.K.2005.Radiation protection by *Terminalia chebula*: some mechanistic aspects. *Mol.Cell Biochem.*277 (2): 43-48

Gilliland, T., Klesius, P.H. and Giambrone, J. 1982. Inhibition of chicken leukocyte migration *in vitro*: a direct agarose plate assay. *Comp. Immunol. Microbiol. Infect. Dis.* 5(4): 457-468

Goldsby, R.A., Kindt, T.J., Osborne, B.A. and Kuby, J. 2002. *Immunology*. Fifth edition. New York, NY: W. H. Freeman. 603p

Gore, A.B. and Qureshi, M.A. 1997. Enhancement of humoral and cellular immunity by vitamin E after embryonic exposure. *Poult. Sci.* 76: 984-991

Gowri, B.S., Platel, K., Prakash, J. and Srinivasan, K. 2001. Influence of amla fruits (*Emblica officinalis*) on the bio-availability of iron from staple cereals and pulses. *Nutr.Res.* 21(12): 1483-1492

Graczyk, S., Kuryszko, J. and Madej, J. 2003. Reactivity of spleen germinal centers in immunized and ACTH treated chickens. *Acta. Vet. Brno.* 72: 523-531

Gupta, S.K. and Bhattacharya, A. 1983. Cytotoxity assays. *A handbook of practical immunology*, edited by Talwar, G.P. Vikas Publishing House, New Delhi. 328

Guruvayoorappan, C. and Kuttan, G. 2007. Effect of *Biophytum sensitivum* on cell mediated immune response in mice. *Immunopharmacol. Immunotoxicol.* 29: 1-14

Hangalapura, B.N., Nieuwland, M.G., Buyse, J., Kemp, B., Parmentier., H.K. 2004. Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult. Sci.* 83(10): 1644-1649

Hanumanthachar, J. and Milind, P. 2006. Brahmi rasayana improves learning and memory in mice. *Evidence based Complementary and Alternative Medicine.* 3(1): 79-85

Hayashi, O. and Kikuchi, M. 1989. Time relationship between ambient temperature change and antigen stimulation on immune responses of mice. *Int. J. Biometerol.* 33(1): 19-23

Jacob, R.A. 1995. The integarated antioxidant system. *Nutr. Res.* 15: 755-766

Jayaprakasan, V. 1986. Cellular and humoral immune responses to *Corynebacterium pseudotuberculosis* infecton in goats. Ph.D thesis, Kerala Agricultural University, Thrissur

Joseph, C.D, Praveen, K.V., Kuttan, G. and Kuttan, R. 1999. Myeloprotective effect on non-toxic indigenous preparation rasayana in cancer patients receiving chemotherapy and radiation therapy. *J. Exp. Clin. Cancer Res.* 18: 325-329

Karthiayini, K. 2007. Effect of season and antistress agents on physiological and biochemical parameters of broiler chicken under stress. Ph.D thesis, Kerala Agricultural University, Thrissur

Khandelwal, S., Shukla, J.L. and Shanker, R. 2002. Modulation of acute cadmium toxicity by *Emblica officinalis* fruit in rat. *Indian J. Exp. Biol.* 40(5): 564-570

Kimball, J.W. 1990. *Introduction to immunology.* Third edition. Macmillan Publishing Co., New York. 523p

Kishimoto, T., Soda, R. Takahashi, K.and Kimura, I. 1987. The role of basophils and mast cells in cutaneous basophil hypersensitivity reaction. *Clin. Exp. Immunol.* 67(3): 611–616

Kitabchi, A.E. 1967. Ascorbic acid in steroidogenesis. *Nature London.* 35: 1385-1386

Kline, K. and Sanders, B.G. 1984. Suppression of ConA mitogen- induced proliferation of normal spleen cells by macrophages from chickens with hereditary muscular dystrophy. *The J. Immunol.* 132(6): 2813-2819

Koenen, M.E., Boonstra-Blom, A.G. and Jeurissen, H. 2002. Immunological differences between layer- and broiler- type chickens. *Vet. Immunol. Immunopathol.* 89(1-2): 47-56

Koren, H.S. and Kim, Y.B. 1978. Natural killing and antibody-dependent cellular cytotoxicity are independent immune functions in the Minnesota miniature swine. *Proc. Natl. Acad. Sci. USA.* 75(10): 5127-5131

Krieger, D.T. 1983. Brain peptides: what, where and why? *Science*.222: 925-985

Kulkarni, S. and Varshney, V.P. 2005. Enhanced adrenocorticotropic hormone and nitric oxide production from caprine peripheral lymphocytes during heat stress. In: Compendium of National Symposium on Recent advances in cryopreservation of livestock germplasm. XIVth Annual Conference of Society of Animal Physiologists of India

Kumar, V.P., Kuttan, R. and Kutta, G. 1999. Effect of "rasayanas" a herbal drug preparation on cell-mediated immune response in tumor bearing mice. *Indian J. Exp. Biol.* 37(1): 23-26

Kuttan, G and Kuttan, R. 1992. Immunological mechanism of action of the tumor reducing peptide from mistletoe extract (NSC 635089) cellular proliferation. *Cancer Lett.* 66 (2): 123-130

Lee, H.S., Won, N.H., Kim, K.H., Lee, H., Jun, W. and Lee, K.W. 2005. Antioxidant effects of aqueous extract of *Terminalia chebula* in vivo and in vitro *Biol. Pharm. Bull.* 28(9): 1639-1644

Lee, I.Y., Lee, C.C., Chang, C.K., Chien, C.H. and Lin, M.T. 2007. Sheng Mai San, a chinese herbal medicine, protects against renal ischaemic injury during heat stroke in the rat. *Clin. Exp. Pharmacol. Physiol.* 32(9): 742-748

Lessard, M., Hutchings, D.L. and Spencer, J.L. 1995. Cell-mediated and humoral immune responses in chickens infected with *Salmonella typhimurium*. *Avian Dis.* 39(2): 230-238

Letwin, B.W. and Quimby, F.W. 1986. Effects of concanavalin A, phyohemagglutinin, pokeweed mitogen and lipopolysaccharide on the replication and immunoglobulin synthesis by canine peripheral blood lymphocytes *in vitro*. *Immunol. letters.* 14: 79-85

Lillehoj, H.S. and Chai, J.Y. 1988. Comparative natural killer cell activities of thymic, bursal, spleenic and intestinal intraepithelial lymphocytes of chickens. *Dev. Comp. Immunol.* 12(3): 629-643

Lin, H., Decuypere, E. and Buyse, J. 2006. Acute heat stress induces oxidative stress in broiler chickens *Comp. Biochem. Physiol. Integr. Physiol.* 144(1): 11-17

Lowenthal, J.W., Connick, T.E., McWaters, P.G. and York, J.J. 1994. Development of T cell immune responsiveness in the chicken. *Immunol. Cell Biol.* 72(2): 115-122

Luger, D., Shinder, D. and Yahav, S. 2003. Hyper or hypothyroidism; its association with the development of ascites syndrome in fast growing chickens. *Gen.Comp.Endocrinol.*127: 293-299

Manu, K.A. and Kuttan, G. 2007. Effect of punarnavine, an alkaloid from *Boerhaavia diffusa,* on cell-mediated immune responses and TMP-1 in B16F-10 metastatic melanoma- bearing mice. *Immunopharmacol. Immunotoxicol.* 29: 569-586

Mashaly, M.M., Hendricks, G.L. 3rd ., Kalama, M.A., Gehad, A.E., Abbas, A.O. and Patterson, P.H. 2004. Effect of heat stress on production parameters and immune response of commercial laying hens. *Poult. Sci.* 83(6): 889-894

Mattingly, D. 1962. A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. *J. Clin. Path.* 15: 374

McDowell, L.R. 1989. *Vitamin in Animal Nutrition*: Comparative Aspects to Human Nutrition. Academic Press, London. 298–322-365–387p

McKee, J.S., Harrison, P.C. and Riskowski, G.L. 1997. Effects of supplemental ascorbic acid on the energy conversion of broiler chicks during heat stress and feed withdrawal. *Poult. Sci.* 76(9): 1278-1286

Mehra, E. and Vaidya, M.C. 1993. *A hand book of practical and clinical immunology,* edited by Talwar, G.P. and Gupta, S.K. CBS publishers, New Delhi

Mehmet, A.V.C., Yerturk, M. and Kaplan, O. 2005. Effect of ascorbic acid on the performance and some blood parameters of Japanese quails reared under hot climate conditions. *Turk. J. Vet. Anim. Sci.*.29:829-833

Miller, L. and Qureshi, M.A. 1992a. Induction of heat-shock proteins and phagocytic function of chicken macrophage following *in vitro* heat exposure. *Vet. Immunol. and Immunopathol.* 30: 179-191

Miller,L. and Qureshi, M.A. 1992b.Comparison of heat-shock and lipopolysaccharide induced protein changes and tumoricidal activity in chicken mononuclear cell line. *Poult. Sci.* 71: 979–987

Misawa, E., Sakurai. T., Yamada, M., Hayasawa. H. and Motoyoshi, K. 2000. Effects of macrophage colony-stimulating factor and interleukin-2 administration on NK1.1+cells in mice. *Int. J. Pharmacol.* 22: 967-971

Mohandas Rao, K.G., Muddanna Rao, S. and Gurumadhva Rao, S. 2006. Centella asiatica (L.) leaf extract treatment during growth spurt period enhances hippocampal CA3 neuronal dendritic arborisation in rats. *Evidence based Complementary and Alternative Medicine.* 3(3): 349-357

Moretta, L., Bottino, C., Pende, D., Mingari, M.C., Biassoni, R. and Moretta, A. 2002. Human natural killer cells: their origin, receptors and function. *Eur. J. Immunol.* 32: 1205-1211

Morgensen, S.C. 1979. Role of macrophages in natural resistance to virus infections. *Microbiol. Rev.* 43: 1-26

Mustafa, A.S. 1992. In *A Hand book of Practical and Clinical Immunology*. Vol.1, edited by G.P.Talwar and S.K.Gupta, CBS Publishers, New Delhi, 270p

Na, M., Bae, K., Kang, S.S., Min, B.S., Yoo, J.K., Kamiryo, Y., Senoo, Y., Yokoo, S. and Miwa, N. 2004. Cytoprotective effect on oxidative stress and inhibitory effect on cellular aging of *Terminalia chebula* fruit. *Phytother. Res.* 18(9): 737-741

Naik, G.H., Priyadarsini, K.I., Naik, D.B., Gangabhagirathi, R. and Mohan, H. 2004. Studies on the aqueous extract of *Terminalia chebula* as a potent antioxidant and a probable radioprotector. *Phytomed.* 11(6): 530-538

Neldon- Ortiz, D.L. and Qureshi, M.A. 1992. The effects of direct and microsomal activated aflatoxin B1 on chicken peritoneal macrophages *in vitro Vet. Immunol. Immunopathol.* 31: 61-76

Niu, K.C., Lin, K.C., Yang, C.Y. and Lin, M.T. 2007. Protective effects of α-tocopherol and mannitol in both circulatory shock and cerebral ischaemia injury in rat heat stroke. *Clin. Exp. Pharmacol. Physiol.* 30(10): 745-751

Niwano, Y., Becker, B.A., Mitra, R., Cldwell, C.W., Abdalla, E.B. and Johnson, H.D. 1990. Suppressed peripheral blood lymphocyte blastogenesis in pre-and postpartal sheep by chronic heat-stress, and suppressive property of heat-stressed sheep serum on lymphocytes. *Dev. Comp. Immunol.* 14: 139-149

Oladele, B.S., Ogundipc, S., Ayo, J.O. and Esievo, N.A. 2001. Effects of season and sex on packed cell volume, haemoglobin and total proteins of indigenous pigeons in Zaria, Northern Nigeria. *Vet. Archiv.* 71: 277-286

Oppenheim, J.J., Ruscetti, E W. and Faltynek, C. R. 1987. Interleukins and interferons. In: Stites, D. P., Stobo, J. D. and Wells, J. V. eds. Norwalk, CT.

Appleton and Lange, *Basic and clinical immunol.*: 82-95

Ota, H. and McNally, E.H. 1961. Engineering in broiler housing – some problems affected by climate. *Argic. Engng.* 42: 616-617

Pardue, L.S., Thaxton, P.J. and Brake, J. 1985. Role of ascorbic acid in chicks exposed to high environmental temperature. *J. Appl. Physiol.* 58 (5): 1511-1516

Parida, R.N.S., Bisoi, P.C., Sahoo, P.K. and Mohapatra, M. 1995. Protective effect of livol during experimental aflatoxicosis in chicks: A histomorphological approach. *Indian J. Indg . Med.* 17(1): 19-25

Parmentier, H.K., De VriesReilingh, G. and Nieuwland, M.G.B. 1998. Kinetic and immunohistochemical characteristics of mitogen-induced cutaneous hypersensitivity in chicks selected for antibody responsiveness. *Vet. Immunol. Immunopathol.* 66: 367-376

Pawan, K., Agrawal, Donald, L. and Reynolds. 1991. Evaluation of the cell-mediated immune response of chickens vaccinated with Newcastle disease virus as determined by the under- agarose leukocyte- migration- inhibition technique. *Avian Dis.* 35(2): 360-364

Perlman, H.P., Perlman, R.D., Schreiber and Miiller-Eberhard, H.J. 1981. Interaction of target cell bound C3bi and C3d with human lymphocyte receptors: enhancement of antibody mediated cellular cytotoxicity. *J. Exp. Med.* 153: 1592

Peterson, A.L., Qureshi, M.A., Ferket, P.R. and Fuller Jr, J.C. 1999. *In vitro* exposure with β-hydroxy- β-methybutyrate enhances chicken macrophage growth and function. *Vet. Immunol. Immunopathol.* 67: 67-78

Ptak, W., Geba, G.P. and Askenase, P.W. 1991. Initiation of delayed-type hypersensitivity by low doses of monoclonal antibody. Mediation by serotonin

and inhibition by histamine. *J. Immunol.* 146: 3929-3936

Puthpongsisriporn, U., Scheideler, S.E., Sell, J.L. and Beck, M.M. 2001. Effects of vitamin E and C supplementation on performance, *in vitro* lymphocyte proliferation, and antioxidant status of laying hens during heat stress. *Poult. Sci.* 80: 1190-1200

Puvadolprid, S. and Thaxton, J.P. 2000.Model of Physiological stress.1.Response parameters.*Poult.Sci.*79: 363-369

Qureshi, M.A., Dietert, R.R. and Bacon, L.D. 1986. Chemotactic activity of chicken blood mononuclear leukocytes from 151_5-B-congenic lines to bacterially derived chemoattractants. *Vet. Immunol. Immunopathol.* 19: 351-360

Qureshi, M.A. and Miller, L. 1991. Signal requirements for the acquisition of tumoricidal competence by chicken peritoneal macrophages. *Poult. Sci.* 70: 530-538

Qureshi, M.A., Kidd, M.T. and Ali, R.A. 1995. *Spirulina platensis* extract enhances chicken macrophage functions after *in vitro* exposure. *J. Nutr. Immunology.* 3: 35

Qureshi, M.A. 1998. Role of macrophages in avian health and disease. *Poult. Sci.* 77: 978-982

Radwan, A.I., Buening, G.M. and Loan, R.W. 1975. ^{51}Cr release microassay for measuring cell mediated immunity in chickens. *Infection and Immunity.*11 (3): 436-440

Ramnath, V., Kuttan, G., Kuttan, R. 2006. Effect of abrin on cell-mediated immune responses in mice. *Immunopharmacol. Immunotoxicol.* 28: 259-268

Ramnath, V., Rekha, P.S. and Mejo, K.R. 2007. Effect of brahma rasayana (BR) supplementation in heat stressed chickens on certain haematological and biochemical variables. *Indian. J. Anim. Sci.* 77(2): 42-46

Ramnath, V., Rekha, P.S. and Sujatha, K.S. 2008. Amelioration of heat stress induced disturbances of antioxidant defense system in chicken by brahma rasayana. *Evidence based complementary and alternative medicine*. 5(1): 77-84

Regnier, J.A. and Kelley, K.W. 1981. Heat- and cold stress suppress *in vivo* and *in vitro* cellular immune responses of chickens. *Am. J. Vet. Res.* 42(2): 294-299

Rekha, P.S., Girija, K. and Ramadasan, K. 2000. Effect of herbal preparation: Brahma Rasayana in the amelioration of radiation induced damage. *Indian J. Exptl. Biol.*37: 999-1002

Rekha, P.S., Kuttan, G. and Kuttan, R. 2001. Effect of brahma rasayana on antioxidant systems and cytokine levels in mice during cyclophosphamide administration. *J. Exp. Clin. Cancer. Res.* 20(2): 219-223

Rekha, P.S., Kuttan, G. and Kuttan, R. 2001a. Effect of brahma rasayana on antioxidant system after radiation. *Indian J. Exp. Biol.* 39(11): 1173-1175

Rekha, P.S., Kuttan, G, and Kuttan, R. 2003b. Role of brahma rasayana on the immune status in irradiated mice. *Indian J. Exp. Biol.* 38: 999-1002

Retsky, K.L. and Frei, B. 1995. Vitamin C prevents metal ion dependent initiation and propagation of lipid peroxidation in human low-density lipoprotein. *Biochem. Biophys. Acta.* 1257: 279-287

Ricci, M., Romagnani, S., Passaleva, A. and Biliotti, G. 1969. Lymphocyte transformation and macrophage migration in guinea- pigs immunized with freund's complete adjuvant. *Clin. Exp. Immunol.* 5: 659-667

Roy, R., Maiti, S.K., Ali, S.L. and Raju, S. 1996. Study on the efficacy of zeetress, an antistress in layers during summer. *Indian Vet. J.* 73: 662-664

Sairam, K., Rao, C.V., Babu, M.D., Kumar, K.V., Agrawal, V.K. and Goel, R.K. 2002. Antiulcerogenic effect of methanolic extract of *Emblica officinalis*: an experimental study. *J. Ethnopharmacol.* 82:1-9

Sairam, M., Neetu, D., Deepti, P., Vandana, M., Ilavazhagan, G., Kumar, D. and Selvamurthy, W. 2003. Cytoprotective activity of Amla *(Emblica officinalis)* against chromium (VI) induced oxidative injury in murine macrophages. *Phytother. Res.* 17: 430-433.

Saxena, K.C., Puri, A., Sumati., Saxena, R. And Saxena, R.P. 1991. Macrophage migration as an index of immune status. *Immunol. Invest.* 20(5-6): 431-440

Scartezzini, P., Antognoni, F., Raggi, M.A., Poli, F. and Sabbioni, C. 2006. Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of *Emblica officinalis* Gaertn. *J.Ethnopharmacol.* 104 (2): 113-118

Senyk, G. and Hadley, W.K. 1973. in vitro correlates of delayed hypesensitivity in man: Ambiguity of polymorphonuclear neutrophils as indicator cells in leukocyte migration test. *Infection and Immunity.* 8(3): 370-380

Sharma, P.V. 1987. *Dravyaguma Vijnam.* Medhya Varga. Chaukambha Bharati Academy, Varanasi

Sharma, J.M. and Lee, L.F. 1983. Effect of infectious bursal disease on natural killer cell activity and mitogenic response of chicken lymphoid cells: Role of adherent cells in cellular immune system. *Infection and Immunity.* 42(2): 747-754

Sheeja, K. and Kuttan, G. 2007. Modulation of natural killer cell activity, antibody-dependent cellular cytotoxicity, and antibody-dependent complement-mediated cytotoxicity by andrographolide in normal and Ehrlich ascites carcinoma- bearing mice. *Integr. Cancer Ther.* 6(1): 66-73

Shephard, R.J. and Shek, P.N. 1998. Cold exposure and immune function. *Can. J. Physiol. Pharmacol.* 76(9): 828-836

Shimizu, K. 1970. Effects of ascorbic acid on the side chain cleavage of cholesterol. *Biochem. Biophysiol. Acta.* 210: 333-340

Shinder, D., Luger, D., Rusal, M., Rzepakovsky, V., Bresler, V. and Yahav, S. 2002. Early age cold conditioning in broiler chickens (*Gallus domesticus*): thermotolerance and growth responses . *J. Thermal Biol.* 27(6): 517-523

Siegel, H.S. 1980. Physiological stress in birds. *Bioscience.* 30: 529-534.

Siegel, H.S. 1995. Stress, strains and resistance. *Br.Poult. Sci.* 36: 3–22

Sijben, J.W.C., Schrama, J.W., Nieuwland, M.G.B. and Parmentier, H.K. 2000. Immunomodulatory effects of indomethacin and prostaglandin E2 on primary and secondary antibody responses in growing layer hens. *Poult. Sci.* 79: 949-955

Silanikove, N. 1992. Effects of water scarcity and hot environment on appetite in animals experiencing severe dehydration in ruminants: a review. *Livest. Prod. Sci.* 30: 175-194

Snedecor, G.W and Cochran, W.G.1994. *Statistical Methods.* Eighth edition. Oxford and IBH publishing corporation..,Calcutta, 564p

Spehner, V., De Wazieres, B., Nicod, L., Harraga, S., Robert, J.F., Seilles, E. 1996. Auditory stress induces changes in membrane functions of mouse peritoneal macrophages. *Scand. J. Immunol.* 44 (6): 643-647

Srikumar, R., Jeyaparthasarathy, N., Sheeladevi, R. 2005. Immunomodulatory activity of triphala on neutrophil functions. *Biol. Pharm. Bull.* 28(8): 1398-1403

Suchalatha, S. and Shyamaladevi, C.S. 2004. Protective effect of *Terminalia chebula* against experimental myocardial injury induced by isoproterenol. *Indian J. Exp. Biol.* 42(2): 174-178

Sujarani, S. 2003. Evaluation of the immunomodulatory effect of *Emblica officinalis* (AMLA) fruit pulp extracts in mice. M.V.Sc. thesis, Kerala Agricultural University, Thrissur

Suresh, K. and Vasudevan, D.M. 1994. Augmentation of murine natural killer cell and antibody dependent cytotoxicity activities by *Phyllanthus emblica*, a new immunomodulator. *J. Ethnopharmacol.* 44: 55-60

Sykes, A. H. 1978. Vitamin C for poultry: Some research. Proceedings of the Roche Symposium

Thaxton, P. 1978. Influence of temperature on the immune responses of birds. *Poult. Sci*. 57: 1430-1440

Thejass, P. and Kuttan, G. 2006. Augmentation of natural killer cell and antibody-dependent cellular cytotoxicity in Balb/c mice by sulforaphane, a naturally occurring isothiocyanate from broccoli through enhanced production of cytokines IL-2 and IFN- γ. *Immunopharmacol. Immunotoxicol.* 28(3): 443-457

Tizzard, I.R. 2004. *Veterinary immunology an introduction.* Seventh edition. Saunders, Philadelphia. 494p

Trout, J.M. and Mashaly, M.M. 1995. Effects of *in vitro* corticosterone on chicken T- and B- lymphocyte proliferation. *Br. Poult. Sci.* 36(5): 813-820

Walker, W.S. and Demus, A. 1975. Antibody-dependent cytolysis of chicken erythrocytes by an *in vitro*- established mouse peritoneal macrophages. *The J. Immunol.* 114: 765-769

Wheeler, G.E. 1994. Use of a herbal supplement to reduce the effects of stress in intensively housed chickens. *Indian J. Indg. Med.* 11(1): 51-60

Widerman, R.F., Ford, B.C., May, J.D. and Lott, B.D. 1994. Acute heat acclimation and kidney function in broilers. *Poult. Sci.* 73(1): 75-88

Wodnar-Filipowicz, A. and Kalberer, C.P. 2006. Functions of natural killer cells in immune defense against human leukemia. *Swiss Med. Wkly.* 136: 359-364

CELL MEDIATED IMMUNE RESPONSE IN COCKERELS UNDER TEMPERATURE STRESS

SAVITHA. M. K.

Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

2008

Departmert of Veterinary Physiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ABSTRACT

A study was conducted with the objective of finding the variations in the cell mediated immunological responses induced by heat / cold stress in cockerels and the role of brahma rasayana (BR) supplementation as an immunopotentiative agent. A total of 144 Gramapriya cockerels having 1 kg bw (4 months) were subjected to heat (40 ± 1^0C and relative humidity (RH) 80 ± 5 per cent) and cold (4 ± 1^0C and RH 45 ± 5 per cent) each for 4 consecutive hours per day for 10 days in a controlled environmental chamber (floor space 875 cm^2 / bird). Control groups were reared randomly under ambient temperature of 30 ± 1^0C and RH 65 per cent. The dietary inclusion of BR was done @ 2.0 g/kg bw for 20 days (10 days prior to and during the period of heat / cold stress).

To certain extend the heat stress induced hyperthermia in birds. Heat stress was positively correlated with the plasma corticosterone level in untreated cockerels, whereas exposure to cold and BR treatment resulted in lowered plasma corticosterone concentration.

The phagocytic potential and migratory capability of peritoneal macrophages was found to be enhanced in both heat / cold stress, which were further enhanced by BR supplementation, while the cytotoxicity against L929 cells was decreased in heat / cold stressed cockerels, which were reversed by BR supplementation.

It was noticed that the cutaneous delayed hypersensitivity response in heat / cold stressed untreated cockerels at 24 / 48 h post PHA inoculation got increased by BR supplementation. Similarly, the leucocyte migration inhibition index which was decreased in temperature stress could be reversed by BR treatment.

Administration of BR was found to enhance the proliferation of splenocytes in response to mitogens like PHA and Con A in both heat / cold stress. The mitogenic response of PHA was dose dependent, while that of Con A was not dose specific.

The natural killer cell activity was found decreased by heat / cold stress and enhanced with the supplementation of BR, earlier lysis of target K562 cells were achieved by effector cells.

The antibody dependent cellular cytotoxicity was enhanced in heat / cold stressed cockerels administered with BR on the ninth day, when compared to their untreated counterparts, which was measured from the percentage SRBC lysis.

An earlier antibody dependent complement mediated cytotoxicity was observed on 5th day of the experiment, while the BR supplementation could augment the tumoricidal property through ACC in heat / cold stressed and non-stressed cockerels.

The cockerels under induced heat stress exhibited much diminished cell mediated immune profile when compared to cold stressed and non-stressed birds. In general, most of the immune parameters screened in cockerels under induced cold stress were found to be in par with non-stressed BR treated group. This indicated that there was not much alteration in the immune status of cockerels during cold exposure. Results of the present study also indicated that the dietary supplementation of BR @ 2.0 g/kg bw in poultry could potentiate the immunological insult inflicted by heat stress.