EFFECT OF GOOSEBERRY (*Emblica officinalis*) AND INDIAN GALL NUT (*Terminalia chebula*) ON THE IMMUNE RESPONSE IN COCKERELS UNDER INDUCED HEAT AND COLD STRESS

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

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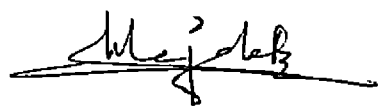
DECLARATION

I hereby declare that this thesis, entitled **"EFFECT OF GOOSEBERRY (*Emblica officinalis)* AND INDIAN GALL NUT *(Terminalia chebula)* ON THE IMMUNE RESPONSE IN COCKERELS UNDER INDUCED HEAT AND COLD STRESS"** is a bonafide record of research work done by me during the course of research and that the 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.



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CERTIFICATE

Certified that this thesis entitled **"EFFECT OF GOOSEBERRY *(Emblica officinalis)* AND INDIAN GALL NUT *(Terminalia chebula)* ON THE IMMUNE RESPONSE IN COCKERELS UNDER INDUCED HEAT AND COLD STRESS"** is a record of research work done independently by **Mejo K.R**, under my guidance and supervision and that it has not previously formed the basis for the award to me of any degree, diploma, associateship to her.

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CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

Stress is a physiological response of an animal to several exogenous and endogenous stimuli (stressors) that cause neuroendocrine activation. An animal is said to be in the state of ***"stress"*** if it is required to make some abnormal or extreme adjustments in its physiology or behavior to cope with adverse aspects of its environment (Fraser and Broom, 1990). There are two main types of reactions to a stressor, viz., the alarm or emergency reaction and the general adaptation mechanism. Corticotropin releasing factor (CRF) is released from the median eminence of the hypothalamus in response to stress that stimulates the synthesis of adrenocorticotropic hormone (ACTH), which in turn makes the adrenal cortex to secrete glucocorticoids that brings about immunosuppressive activities (Negra *et al.*, 1962). Similarly, during stress lymphocytes produce ACTH, which has a powerful immune suppressor activity independent of its capacity to stimulate glucocorticoid secretion (Stefano and Smith, 1996).

In tropical and arid areas, environmental temperature is the major stress factor (Silanikove, 1992). Heat stress destabilizes the animal's energy, water, hormonal and mineral balances thereby adversely affect the performance and well-being of livestock including poultry (Hahn, 1999). Since the range of comfort zone or thermoneutral zone of birds being narrow, they are highly susceptible to heat and cold stress.

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 which not only act against stress causing agents, but also improve defense function. Vitamin C plays a pivotal role in decreasing the continued synthesis of corticosterone from adrenal cortex. Although poultry can synthesize vitamin C, synthesis becomes inadequate under stressful conditions (Sykes, 1978; McDowell, 1989).

Several methods are available to alleviate negative effects of high environmental temperature on performance of poultry. Because of the high cost

and impracticability of cooling animal sheds, interest on dietary manipulations has increased. One of the main strategies in using herbal products is to increase body's natural resistance to pathogens rather than directly neutralizing the agents itself.

The traditional Indian system of medicine namely Ayurveda has given great emphasis on the promotion of health. Rasayanas are a group of non-toxic single or polyherbal preparation commonly used in indigenous medical practice in India to improve the health and longevity. Brahma Rasayana (BR) and Chyavanaprasha (CVP) are two non toxic poly herbal preparation, made from the extracts of plants, claimed to be potent immunomodulators and widely used as rejuvenating health tonic to vitalize the immune system in human beings (Joseph *et al.*, 1999; Jagatia *et al.*, 2004). Gooseberry (*Emblica officinalis*) and Indian gallnut (*Terminalia chebula*) are the two major ingredients of BR and CVP accounting more than 75% $^{w}/_{w}$ of the preparation (Acharya, 1981).

Paucity of information exists with respect to the usage of immunomodulatory drugs to ameliorate the immunosuppressive effects of excess endogenous corticosteroids produced during stress. The present study was undertaken to determine the physiological changes and immunomodulatory activity of combined supplementation of Gooseberry (GB) and Indian gallnut (IGN) in stressed birds with the following objectives

(a) To find out the various physiological changes brought about by environmental stress (heat and cold) in chicken and the effects of combined supplementation of GB and IGN as the anti-stress agents.

(b) To evaluate the immunological status of chicken under heat and cold stress and the effect of combined supplementation of GB and IGN as an immunopotentiative agent

(c) To explore the possibility of the usage of GB and IGN as adaptogenic agent in chicken during adverse environmental conditions.

Review of Literature

2. REVIEW OF LITERATURE

Under the routine production conditions various types of stress such as heat/cold, transport and preslaughter holding were experienced by chickens. 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 offer an alternative to synthetic compounds and 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.

Fruits of *Emblica officinalis* Gaertn. (Family Euphorbiacea) commonly known as 'Gooseberry' or 'Amla' has been extensively used in Indian Ayurvedic and Sidha systems of traditional medicine in the treatment of wide spectrum of diseases. It is the 2nd highest known source of natural Vitamin C next to acerola berries (*Malpighia glabra* Linn.) and it does not dissipitate either under high heat or reasonable ageing and this is because it is bound up with certain tannins. It is a native plant of north and southwest India. It is the constituent of several marketed herbal preparation such as Chyavanaprasa, Brahma Rasayana, Triphala and Septilin. Some of the properties of the fruit experimentally proved include antifungal, antibacterial, antidiabetic, antipyretic, antioxidant, anticlastogenic and hepatoprotective functions.

Fruits of *Terminalia chebula* (Family Combretaceae) commonly known as 'Indian gallnut', 'Harithika' or 'Myrobalan' and it have been mentioned in the ancient Indian medicinal system (Charaka sanhita, ca 1500 BC). It is the native plant of India and South East Asia. The *T. chebula* has been reported to exhibit astringent, purgative, stomachic and laxative properties.

2.1.PROPERTIES OF GOOSEBERRY (*Emblica officinalis)*

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.

Xia *et al.* (1997) quoted that there were 17 countries in the world that use various parts of *E. 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.

Rekha *et al.* (2000) found that oral administration of "Brahma Rasayana" (10 and 50 mg/dose/animal) that contained *E. officinalis* as one of the components 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.

Khopde *et al.* (2001) stated that ascorbic acid and polyphenols present in the extracts of *Emblica* showed much superior antioxidant activity compared to their equivalent amounts in pure isolated forms.

Pawar and Somkumar (2001) evaluated the antianaemic efficacy of aqueous extract of *E. officinalis* (200 mg/kg bw/day) in rats against anaemia induced by repeated bleeding @ 12 to 15 ml/kg bw for consecutive 5 days. They noticed that mean haemoglobin level increased by 15.89% and mean packed cell volume in treatment group was increased by 18.89% and convinced the potential of *E. officinalis* to correct the anaemia.

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.

Panda *et al.* (2002) studied the effect of *E.officinalis* in regulating thyroid functions in male mice and they found that oral administration of fruit extract @ 30mg/kg bw, each day for 20 days decreased serum T_3 and T_4 concentrations.

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 cytotoxicity and free radical production. The result revealed that GB inhibited apoptosis and DNA fragmentation induced by Cr and relieved the immunosuppressive effects on lymphocyte proliferation.

Panda and Kar (2003) reported that the ethanolic extract from the fruits of *E. officinalis* Gaertn reduced T_3 and T_4 concentrations by 64 per cent and 70 per cent respectively as compared to a standard anti thyroid drug, propyl thiouracil which decreased the levels of the thyroid hormones by 59 and 40 per cent respectively in mice.

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.

Harikumar *et al.* (2004) studied the modulation of haematopoetic system and antioxidant enzymes by *E. officinalis* Gaertn. and its protective role against gamma-radiation induced damages in mice treated with 2.5 g/kg bw of *Emblica* for 10 consecutive days before and after a single dose of 700 rads (7Gy) of radiation. They observed significant increase in the total leukocyte count (TLC), bone marrow viability and hemoglobin (Hb) levels, which were lowered by irradiation. They concluded that the extract significantly reduced the bioeffects of radiation.

Rao *et al.* (2005) studied the antioxidant properties of *E. officinalis* extract and their effects on the oxidative stress in diabetic rats. Gooseberry in the form of extract (20 or 40 mg/kg of body weight/day) was given orally for 20 days to the streptozotocin induced diabetic rats. Gooseberry extracts showed strong free radical scavenging activity. The oral administration of gooseberry extracts to the diabetic rats slightly improved body weight gain and also significantly alleviated oxidative stress indices of the serum like creatinine and 5-hydroxymethylfurfural levels in diabetic rats.

Srikumar *et al.* (2005) assessed the immunomodulatory activities of Triphala (*Terminalia chebula, Terminalia belerica* and *Emblica 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.

2.2.PROPERTIES OF INDIAN GALLNUT (*Terminalia chebula*)

Dabbert *et al.* (1997) studied the properties of *T.chebula* and reported that it exhibited a variety of biological activities including anticancer, antidiabetic, antimutagenic, antifungal and antiviral properties

Na *et al.* (2004) reported that the ethanol extract from the fruit of *T.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 body wt) induced myocardial damage in rats *T. chebula* extract (500 mg/kg body wt) 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. 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. 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 t-butyl hydroperoxide (t-BHP) induced oxidative injury observed in cultured rat primary hepatocytes and rat liver. Both treatment and pretreatment of hepatocytes with the *T. chebula* extract (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.

Parmar *et al.* (2006) reported the effect of *Terminalia arjuna* bark extract in decreasing the level of thyroid hormones and cardio lipid peroxidation, suggesting the possible mediation of the drug action through an inhibition in thyroid function.

2.3.EFFECT OF ENVIRONMENTAL STRESS ON BODY WEIGHT

Pardue *et al.* (1985) conducted a study to determine the role of ascorbic acid in male chickens exposed to high ambient temperature (38°C). They found that heating increased ($P<0.005$) mortality, where as ascorbic acid (AA) (1000 ppm) supplementation improved livability ($P\leq 0.07$). 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 °C over 24h) had no significant effect on body mass, whereas, cold stress (cycled from 3.6 to ~ 20 °C over 24h) resulted in loss of an average 9.1± 0.9 g of body mass and thermoneutral birds (constant 21°C) gained an average of 2.4 ± 1.6 over the four days trial.

McKee *et al.* (1997) studied the effect of supplemental ascorbic acid on the energy conversion of broiler chicks during heat stress (33.40±1°C) and they noticed that heat exposure depressed ($P<0.05$) weight gain, while ascorbic acid supplementation increased ($P<0.10$) 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°C; 70-80% 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 35.00 ± 1°C; 25-30% RH resulted in increased cloacal temperature, 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 ascorbic acid on the performance of Japanese quails reared under hot conditions. They reported that oral administration of ascorbic acid (500.00 mg/kg) had significant gain on body weight and feed consumption until fourth week.

2.4. EFFECT OF ENVIRONMENTAL STRESS ON ADRENAL AND SPLEEN WEIGHT

Pardue *et al.* (1985) conducted a study to determine the role of ascorbic acid in male chickens exposed to high temperature (38 °C). They found that heating increased ($P<0.005$) mortality, where as ascorbic acid (AA) (1000 ppm) supplementation improved livability ($P\leq 0.07$). They also found that heating reduced spleenic weight, but supplemented chicks displayed larger spleenic weight and heating did not affect adrenal weights although AA treated birds possessed smaller adrenals.

Spinu and Degen (1993) explored the effect of cold stress and immune response of Bedouin and White leghorn (WL) hens and found that Bedouin hens were well adapted to arid conditions. Cold winter nights resulted in the involution of lymphoid organs like spleen and a depression of immune function to a greater extent in WL hens when compared to Bedouin hens.

Dabbert *et al.* (1997) studied the effects of acute thermal stress on the immune system of the northern bobwhite (*Colinus virginianus*). They noticed that neither heat stress (cycled from 30.80 to 39.00 °C over 24h) nor cold stress (cycled from 3.60 to ~ 20.00 °C over 24h) for four consecutive days had no significant effect on spleen mass.

Puvadolprid and Thaxton (2000) found that continuous delivery of ACTH at 8.00 IU/kg bw/day for 7 days resulted in the decrease of relative weights of major immunological organs i.e., spleen.

Graczyk *et al.* (2003b) studied the reactivity of spleen germinal centers in SRBC immunized and ACTH treated chickens. They found that after 24h of ACTH injection (0.01 mg/100 g bw) a clear hypertrophy of fascicular and reticular zone of adrenal gland with a simultaneous appearance of lymphatic follicle in the cortex, which proved the functional and morphological mobilization of adrenal cells under the influence of the hormone.

Koko *et al.* (2004) studied the effects of acute heat stress on rat adrenal glands and they found that stress exerts tropic (short term) and trophic (long term) effects on the adrenocortical zona fasciculata (ZF) and zona reticularis (ZR). They noticed tropic effects involved an immediate increase in corticosteroid hormone secretion, 10 min after the beginning of stress and it reached the maximum 15-30 min later. They observed trophic effect of ACTH which involved an increased adrenal mass and in the steroidogenic capacity of adrenocortical cells.

2.5. EFFECT OF ENVIRONMENTAL STRESS ON HAEMATOLOGICAL PARAMETERS

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.

Blake and Banchero (1985) reported that cold acclimated guinea pigs lacked ventilatory response to acute hypoxia, apparently caused the marked erythropoeisis and the increased hematocrit observed throughout chronic exposure to cold. The high blood viscosity resulting from the increased hematocrit contributes to the right ventricular hypertrophy and cardiac failure in guinea pigs chronically exposed to cold.

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.

Dabbert *et al.* (1997) noticed that the total peripheral blood leucocyte counts of northern bobwhite (*Colinus virginianus*) subjected to cold stress (cycled from 3.60 to ~ 20.00 °C over 24h) for four consecutive days were > 30 percent lower ($P<0.017$) than those in the thermoneutral condition. Cold stress brought an increase ($P=0.053$) in the relative percentage of monocytes but decreased ($P=0.037$) 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.

Wessely *et al.* (1997) studied the normal erythroid progenitors of the chicken, to determine the molecular player mechanisms involved in making the decision between self-renewal and differentiation in erythroid progenitors. They identified that the glucocorticoid receptor (GR) acts as a key regulator of erythroid progenitor self-renewal (i.e. continuous proliferation in the absence of differentiation).

Yahav *et al.* (1997) studied the haemodynamic changes in broiler chickens during exposure to constant temperatures (10 to 35° C) or diurnal temperature cycles, and during acute heat or cold. They found that packed cell volume (PCV) was significantly high at low constant temperatures (10° and 15° C), whereas at high temperatures (30° and 35° C) 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. Acute exposure of chickens to high temperature did not affect PV or PCV, but resulted in hyperthermia (44.70 ±0.40° C). 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.

Lindern *et al.* (1999) examined the role of corticosteroids in the *in vitro* expansion of primary erythroid cells in liquid cultures and colony assays. They noticed that dexamethasone, a synthetic glucocorticoid hormone, cooperated with Epo and stem cell factor acted directly on erythroid progenitors and maintained the colony-forming capacity of the progenitor cells expanded in liquid cultures. The hormone also delayed terminal differentiation into erythrocytes, which was assayed by morphology, hemoglobin accumulation, and the expression of genes characteristic for immature cells. They also observed that in avian erythroid progenitors, the steroid hormones acted through tyrosine kinase receptors to induce renewal of erythroid progenitors.

Altan *et al.* (2000) studied the effect of heat stress on some blood parameters in broilers. They found that exposure of broilers to 39.00°C, significantly increased rectal temperature ($P<0.05$), 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.

Puvadolprid and Thaxton (2000) found that administration of ACTH @ 8.00 IU/kg bw/day for 7 days caused significant increase ($P<0.05$) in H/L ratio. A shift to right in H/ L ratio was also pronounced in birds treated with ACTH.

Oladele *et al.* (2001) determined the effect of season and sex on their packed cell volume (PCV), haemoglobin (Hb) and total proteins in pigeons and they found highest PCV and Hb was obtained 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.

Graczyk *et al.* (2003a) conducted studies on haematological and metabolic changes during acute stress in turkeys. They observed that the transportation of birds as well as their immobilization caused a shift in the proportional share of heterophils and increase of H/L ratio. They noticed that in turkeys exposed to transport stress, the value of H/L ratio exceeded 1.1, whereas those birds maintained in farm conditions it was only 0.5 and they concluded that change in H/L ratio was a perfect measure of the response on stressors activity.

Graczyk *et al.* (2003b) 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.

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

2.6.EFFECT OF ENVIRONMENTAL STRESS ON BIOCHEMICAL PARAMETERS

Thomas and George (1975) reported that cold exposure (3.5°C) for 21 days produced no change in the concentration of plasma glucose, plasma free fatty acids, muscle and liver glycogen in adult Japanese quails, but the food consumption increased, and quail maintained their body weight. They found that acute cold exposure (–18°C) for 30 minutes produced hypothermia, marked glycogenolysis in muscle and liver.

Carey *et al.* (1978) reported that fasting winter goldfinches exposed to –10°C for 17 h overnight utilized significant amount of body lipid. They also

observed that total body protein, liver and pectoralis muscle carbohydrate, and pectoralis muscle fatty acids do not differ significantly between control and cold stressed birds.

Davison and Lickiss (1979) studied the effect of cold stress (4h at 10°C) on one day old chicken (*Gallus domesticus*) and stated that increased plasma lactate dehydrogenase (LDH) activity indicated transient changes in muscle membrane permeability during exposure and for 8h afterwards. They also found that creatine kinase (CK) activity remained unchanged.

Sarkar *et al.* (1981) found that low environmental temperature (5°C) resulted in efflux of potassium from rat red blood cells. They also found that blood DPG was significantly enhanced, and they concluded it as an adaptive mechanism during cold exposure.

Pardue *et al.* (1985) studied the role of ascorbic acid in male chickens exposed to high temperature (38°C). 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 heated chicks. They found that heating increased plasma protein and decreased K^+ levels, and they also noticed that plasma cholesterol and Na^+ levels were not affected significantly by either AA or heating treatments.

Dawson *et al.* (1992) reported that the T_4 concentrations higher in summer than in winter and T_3 concentration higher in winter than in summer in American gold finches.

Chandratilleke *et al.* (1994) reported thyroid hormone could influence immune system activity during exposure to cold. They found low *in vitro* doses (0.10 ppm) of T_3 enhanced production of IL-2 like activity in birds, but higher (1.00 ppm) doses were found to be immunosuppressive.

Bogin *et al.* (1996) studied the metabolic changes leading to death of the domestic chicken during acute heat stress. They observed that significant increase in the blood levels of glucose, uric acid, calcium, potassium, T_3, and erythrocyte creatine kinase (CK) contents.

Bohek *et al.* (1996) analyzed the role of reverse triiodothyronine (rT_3) in heat stressed immature chickens. When injected subcutaneously, rT_3 (14 mg/100 kg bw) aggrevated heat stress symptoms and increased plasma levels of corticosterone, catecholamines, and free fatty acids in chickens.

Dabbert *et al.* (1996) determined serum biochemistry of 120 adult male Northern bobwhite (*Colinus virginianus*) maintained either at thermoneutrality or exposed to thermal stress. They noticed that lactate dehydrogenase and uric acid in serum of cold stressed birds were greater compared to thermoneutral or heat-stressed birds and serum total protein was greater in cold stressed birds than in heat-stressed birds.

Dabbert *et al.* (1997) studied the effects of acute thermal stress (heat stress cycled from 30.8 to 39.0 °C over 24h) and cold stress (cycled from 3.6 to ~ 20 °C over 24h) for four consecutive days on the immune system of the northern bobwhite (*Colinus virginianus*). They found that serum T_4 concentrations were 27 per cent lower (P=0.009) in cold stressed than in birds maintained at thermoneutrality, but T_3 concentrations were similar between treatments.

Yahav *et al.* (1997) examined the broiler chicks exposed to 36 ±1°C; 70-80% relative humidity (RH) for 24 h at the age of 5 days and they found that on conditioning at an early age, the chicks exhibited reduced plasma triiodothyronine (T_3) concentration and hemodynamic changes like significant decrease in heart weight and haematocrit.

Terblanche and Nel (1998) investigated the effects of heat acclimation on creatine kinase in serum of male Sprague- Dawley rats. They acclimation at 33±1°C resulted in significantly lower levels of CK activity.

Gabay and Kushner (1999) stated that the C-reactive protein as aprototype acute phase reactant, and its plasma level increased by as much as 1000 fold from a normal value of less than 2μg/ ml with in hours of onset of most tissue damaging inflammatory processes.

Puvadolprid and Thaxton (2000) found that continuous delivery of ACTH at 8.00 IU/kg bw/day for 7 days significantly $P<0.05$) increased plasma corticosterone, cholesterol, and total protein levels.

Sandercock *et al.* (2001) studied the effect of acute heat stress and found that it would lead to increased deep body temperature, panting induced acid/base disturbances and plasma CK activity, reflecting heat stress induced myopathy.

Burger and Denver (2002) studied the relationship of plasma thyroid hormone concentrations in a wintering passerine bird with environmental factors, geographic variation and metabolic rate. They measured total plasma T_4 and T_3 concentrations in birds during two latitudinal transect extending from 31°N to 42 °N. They found that birds from both transects had higher plasma thyroid hormones in the later afternoon than in early morning and plasma T_3 increased with latitude, while plasma T_4 varied such that birds in intermediate latitude had lesser T_4. They concluded that higher temperatures are associated with lower plasma T_3 concentrations and plasma T_4 concentration was positively correlated with temperature variables.

Shinder *et al.* (2002) examined the influence of short repetitive cold exposure at an early age and at 21 days of age (cold conditioning-exposure to 15°C for 3 h at 3 and 4 days of age) on chickens' thermotolerance. They noticed that cold exposure elicited a dramatic decline in body temperature and a significant elevation in plasma corticosterone concentration and upon chronic exposure it was remained at a significantly lower level. Also observed that conditioned chickens exposed to optimal conditions (22°C) achieved significantly

higher body weights than others and hence early cold conditioning improves thermotolerance in broiler chickens in later life.

Borges *et al.* (2003) evaluated the effect of heat stress in individually caged male Cobb broilers, 44 d of age and the temperature sequence for heat stress was 24 to 32°C in 30 min, 32 to 36 °C in 30 min, 36 to 37° C in 15 min, and 37 to 41°C in 45 min. They noticed that heat stress decreased blood Na, K, and pCO_2, and lymphocytes but increased heterophils.

Djordjevic *et al.* (2003) studied the activation of ACTH and corticosterone release in response to various stressors in rats. They noticed a significantly ($P<0.05$) increased ACTH plasma levels in rats exposed to different stressors (38°C, 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 found that the corticosterone concentration was markedly increased in the serum of all the stressed animals as compared to the controls, which confirmed the intense of glucocorticoid secretion in response to stressors. They concluded that exposure to ambient temperature of 38°C appears to be the strongest stressor activating the HPA system.

Moreas *et al.* (2003) investigated the effect of thermal conditioning, (through exposure to heat stress), during pre-hatch development on some physiological responses of post-hatch broilers to a post-natal heat stress challenge. They proposed that exposure to heat stress at that stage induced epigenetic heat adaptation and that the thermal conditioning during incubation did not affect the plasma T_4, corticosterone, glucose, uric acid and CK concentrations. Temperature challenge decreased plasma T_3 of broilers of both groups but the decrease was greater in pre-conditioned broilers compared with controls

Nazifi *et al.* (2003) studied the influence of thermal stress on serum biochemical parameters of Iranian fat tailed sheep and they reported the concentrations of total protein, glucose, cholesterol, total lipid, calcium, inorganic

phosphorus, magnesium, creatine kinase (CK), lactate dehydrogenase (LDH), triiodothyronine (T_3), Thyroxine (T_4) in cold (4°C) stress conditions were higher than in heat (40°C) stress and they found that there was no significant difference in cortisol concentrations at either heat or cold stress.

Shirpour *et al.* (2003) investigated the influence of hypothermia on thyroid gland function and its role in metabolic balances in rats (albino Wister race). They reported that TSH levels were altered and found that T_3 increased significantly and T_4 decreased following hypothermia. They also found decreased body temperature after hypothermia.

Wilson (2003) reported that the ratio of sodium to potassium reflects adrenal glandular activity. A ratio greater than 2.5:1 represented a tendency for an excess of pro-inflammatory adrenal hormones such as cortisol and cortisone whereas a low ratio was a chronic stress indicator and it also associated with an impaired immune system. Also stated that acute stress increased adrenal gland activity, leading to increased aldosterone secretion, which resulted in increased retention of sodium in the body causing high level of sodium in the body tissues while the potassium level remained low.

Hangalapura *et al.* (2004) studied the effect of cold stress (CS) on immune response and plasma adrenal and thyroid hormone levels in chickens. They found that the hormonal changes involving corticosterone (CORT), triiodothyronine (T_3), and thyroxine (T_4). No significant effect of duration of CS on specific antibody titers was found in the 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 CORT levels. One day of CS had a significant enhancing effect on T_3 levels. There was no significant effect of duration of CS on T_4 levels. They suggested that CS does not affect specific antibody responses, but may have a modulating effect on cellular immunity and plasma CORT levels, depending on the duration of the stress and concluded that there was an inverse relationship between LP and CORT.

Debut *et al.* (2005) compared the behavioural and physiological responses to acute heat stress in three different chicken breeds. Bird's physiological status was estimated by measuring blood corticosterone, glycaemia, creatine kinase activity, acid-base status and electrolyte concentration as well as lactate content and glycolytic potential in the breast (Pectoralis major) and thigh (Ilio tibialis) muscles. They noticed that acute heat stress affected blood Ca^{2+} and Na^{+} concentration and increased glycaemia and glycolytic potential of thigh muscle.

Collin *et al.* (2005) stated that thyroid hormones were involved in the control of thermoregulation in birds and mammals. They reported that these hormones play a role in the regulation of heat production. The underlying mechanisms could be the stimulation of the nuclear and mitochondrial transcription of several genes involved in energy metabolism and/or a direct action on the activity of components of the mitochondrial respiratory chain. They focused on a subfamily of mitochondrial anion carriers called uncoupling proteins (UCPs). These proteins were suspected to be involved in a partial dissipation of the mitochondrial proton electrochemical gradient that would uncouple phosphorylations from oxidations and hence produced heat. The expression of avian UCP was demonstrated to be strongly regulated by thyroid status in chicken, and over expressed in experimental conditions favouring high triiodothyronine concentrations and thermogenesis.

Kumar *et al.* (2005) studied the effect of C-reactive protein level during experimental mycotoxicosis and its amelioration using antioxidants in broiler chickens. They found that the C-reactive protein (CRP) levels in the serum of aflatoxin fed birds increased ($P<0.05$) on day 14 to 42 and also noticed that a significant decrease in the serum CRP levels in birds continually fed with mycotoxins and antioxidants supplements.

Mehmet *et al.* (2005) reported that oral administration of ascorbic acid (500 mg/kg) on Japanese quails reared under hot conditions did not effect the concentrations of serum Sodium (Na), Potassium (K) or bicarbonate (HCO^{-}_{3}).

Lin *et al.* (2006) investigated the stress response and oxidative damage in plasma, liver and heart in broiler chickens acutely exposed to high temperature. Eighty five-week old broiler chickens were exposed to 32 °C for 6 h. The blood metabolites such as glucose, urate, triiodothyronine (T_3), thyroxine (T_4), corticosterone, ceruloplasmin and creatine kinase (CK) were measured before and after 3 and 6 h of heat exposure. The results showed that oxidative stress could be induced in 5-week old broiler chickens by acute heat exposure (32 °C, 6 h). They suggested that the elevated body temperature could induce the metabolic changes that are involved in the induction of oxidative stress. The liver was more susceptible to oxidative stress than heart during acute heat exposure in broiler chickens.

Sandercock *et al.* (2006) examined the effect of acute heat stress (2 h at 32°C and 75% RH) on body temperature and indices of respiratory thermoregulation and skeletal muscle function in two divergently selected male grandparent lines of broiler and layer-type chickens at two ages (35 and 63 d), or at a similar body weight (approximately 2.2 kg). Plasma creatine kinase (CK) activities reflecting muscle membrane damage were greatly elevated in the broiler line. Exposure to acute heat stress caused an increase in deep body temperature, panting-induced acid-base disturbances and elevated plasma CK activity in both lines of chicken, an effect that increased with age. The extent of disturbances in acid-base regulation and heat-stress-induced myopathy were more pronounced in the broiler than the layer line at the same age or similar live weights.

2.7.EFFECT OF ENVIRONMENTAL STRESS ON IMMUNOLOGICAL PARAMETERS

Reyes and Francois (1971) examined the spontaneous rosettes, obtained with nonimmunized mouse spleen cells and heterologous erythrocytes. They studied the rosettes in detail by electron microscopy, isolated the rosettes by micromanipulation; embedding was performed by a technique suitable for single-cell study. Furthermore they investigated the morphology of these spontaneous

rosette-forming cells (RFC sp.) by experiments where spleen cells were incubated with antilymphocyte serum (ALS) and complement, at a concentration, which inhibits rosette formation *in vitro*. They postulated that population of RFC sp. contains two classes of cells: lymphocytes and macrophages. The lymphocytes appear to have a constant morphology, corresponding to the "inactive" monoribosomal small lymphocyte. The macrophages exhibited a variable degree signs of erythrophagocytosis. They compared the observations with an already known morphology of cells involved in antibody production. The existence of two cell types in the RFC sp. population was discussed in relation to cellular cooperation in the induction of an immunological response to antigens such as heterologous erythrocytes.

Thursh *et al.* (1972) studied the ultrastructure and ^{3}H-thymidine uptake of rosette-forming cells (RFC) for chicken erythrocytes in normal mice, immunized (primed) 110–180 days previously, and in mice undergoing primary or secondary immunization. They found that ultrastructurally most RFC were lymphocytes, though plasma cells, macrophages, and polymorphonuclear (neutrophilic) leucocytes also formed rosettes, especially in recently immunized mice. They reported that no consistent qualitative morphological differences among the lymphocyte rosette-formers of the different groups were found. Quantitative differences with respect to the percentages of inactive, intermediate, and activated lymphocyte rosette-formers were found, however, between the cells of recently immunized mice and those of mice not recently immunized. A similar difference was found in the percentage of RFC incorporating ^{3}H-thymidine in the two groups. Neither the ultrastructural nor the radioautographic studies revealed significant differences between RFC of normal mice and those of primed mice, nor between RFC of mice undergoing a primary response and those of mice undergoing a secondary response.

Tufveson and Alm (1975) studied the cells forming rosettes (RFC) with sheep erythrocytes (SRBC) and rabbit erythrocytes (RRBC) during the ontogeny of the bursa of Fabricius of the chicken. The frequency of SRBC-RFC was low

but significant in the bursa of 15-day-old embryos, and increased thereafter in an approximately linear fashion with the age of the bursa donor until at least in 43-day-old chickens. They found that total number of SRBC-RFC per bursa increased throughout the ontogeny with no evidence for any abrupt increase after hatch and that the mean number of SRBC bound per RFC also increased after hatch. In contrast, they noticed that the frequency of bursal RRBC-RFC was high in 15-day-old embryos, but significantly decreased on embryonic day 18, remained low until at least 7 days after hatch and then increased significantly. The total number of RRBC-RFC per bursa in contrast remained relatively constant during the embryonic period and the first week after hatch and thereafter increased markedly. They also reported that RRBC-RFC was also frequent in the yolk sac, spleen, and thymus of 15-day-old embryos.

Dagg *et al.* (1977) studied the cellular kinetics of the anti-mouse RBC response (anti- MRBC) in chickens. They compared the serum haemagglutinin (HA) titres, spleenic plaque forming cells (PFC) and rosette forming cells (RFC) with either mouse or sheep red blood cells (SRBC). They found that antibody titre was greater for MRBC than SRBC, but the PFC and RFC values were similar. The RFC and PFC levels were maximum at 4 to 5 days after immunization. They compared level of spontaneous and antigen induced RFC for MRBC in several lymphoid tissues and showed that RFC were abundant in the spleen, less frequent in blood, and infrequent in thymus and bursa of immunized birds. They also observed that the number of PFC and RFC in the spleen increased rapidly from the third day after immunization with MRBC and reached the peak concentration at about 5 days.

Subba Rao *et al.* (1977) studied the effect of cold exposure on the immune response of chickens. They observed that chronic cold (7.2 °C) exposure significantly increased the antibody titers, against SRBC, whereas they found that those birds exposed to 32.2 °C and above had significantly depressed agglutinin levels. They also noticed that short term cold exposure (2 or 4 times) following antigen injection enhanced the agglutinin and hemolysin response, and thirty

minute cold exposures for 2 or 4 times significantly increased IgM antibody production and markedly reduced the IgG antibody. They concluded that the elevation of antibody titers were in relation to time of cold treatment and antigen injection. A significant rise in plaque forming cells against SRBC was detected in birds receiving cold treatments, 12h following the antigenic stimulations.

Hirota *et al.* (1980) described a simple plaque method for detecting cells producing antibodies of specific immunoglobulin class in the chicken based upon the inability of chicken antibody to activate guinea-pig complement. IgM- and IgG-specific plaque-forming cells in the spleen of chickens immunized with sheep red blood cells were detected using guinea-pig complement and rabbit anti-chicken-μ- or γ-chain serum. The specificity of the immunoglobulin class of plaques was confirmed by the abolition of class-specific hemolytic plaques after treatment of the lymphoid cells with rabbit antisera specific for chicken heavy chains in the presence of guinea-pig complement.

Henken *et al.* (1982) investigated the effect of environmental temperature on the humoral immune response of pullets following injection of SRBC. They found a significant ($P<0.05$) increase in total haemagglutinin anti-SRBC antibody titres from day 5 after immunization in pullets maintained at 40°C compared to the antibody titres of pullets maintained at 25°C. Also a significant increase in 2-mercaptoethanol resistant antibody titres from day 5 after SRBC injection was noticed.

Pardue and Thaxton (1984) reported the immunosuppressive effects of corticosteroids in chicken and explained the role of supplemented ascorbic acid (AA) in ameliorating the immunosuppression induced by exogenous cortisol. They found that cortisol treated chicks exhibited significantly reduced agglutinins to sheep red blood cells and they found that AA supplementation resulted in higher haemagglutinin tire levels to SRBC and that AA significantly ameliorated the immunosuppressive action in the chicken.

Pardue *et al.* (1985) studied the role of ascorbic acid in male chickens exposed to high temperature (38°C). The anti-sheep red blood cell (anti-SRBC) haemagglitinin levels after challenging with SRBC (1 ml, 10^5 cells, i.v) suggested that heat mediated immunosuppresion was ameliorated by AA supplementation.

Williamson *et al.* (1990) investigated the effect of raising and lowering circulating thyroid hormones in young domestic fowl on antibody production and the responses of peripheral blood lymphocytes (PBL) to the mitogens like concanavalin A (con A) and phytohaemagglutinin (PHA). They found that levels of circulating thyroid hormones were reduced by treatment with the goitrogen, methimazole, and raised by giving implants containing thyroxine (T_4) and/or triiodothyronine (T_3) and raising or lowering circulating thyroid hormones had no significant effect on peak antibody titers, though the decline in titer was slower in goitrogen-treated birds. They suggested that stress-induced changes in thyroid hormones could modulate cell-mediated immune responses.

Denno *et al.* (1994) stated that norepinephrine (NE) and epinephrine (E) functioned as chemical messengers in the central nervous and the endocrine systems of the chicken. They determined the effects of *in vivo* and *in vitro* exposure of NE and E on IgM and IgG spleenic plaque-forming cell (PFC) formation to the antigen SRBC. Six-week-old New Hampshire chickens were injected i.v. with NE (500 μg/kg bw) or E (100 μg/kg bw) followed by 1 ml of 5% SRBC 30 min later. Five days after antigen injection, IgM and IgG PFC were assayed. They found that when compared with controls, *in vivo* NE treatment had suppressed ($P < 0.05$) IgM and IgG PFC formation. *In vitro* NE treatment of spleenic lymphocytes reduced ($P < 0.05$) IgM PFC but did not affect IgG PFC numbers. *In vivo* treatments with E increased ($P < 0.05$) IgM PFC whereas *in vitro* E exposure increased ($P < 0.05$) IgM PFC. Immunoglobulin G PFC were suppressed ($P < 0.05$) by both *in vivo* and *in vitro* E exposure. The presence of surface receptors for NE and E on spleenic lymphocytes was determined using *in vitro* incubation with antagonists to alpha and beta receptors. They suggested that

there were alpha and beta receptor sites on lymphocytes for NE and E, and that these catecholamines had a regulatory role in plaque cell proliferation.

Bhatnagar *et al.* (1996) explained the bidirectional nature of the communication between the immune and hypothalamic-pituitary-adrenal (HPA) systems in rats, and examined whether those differences in HPA responses to stress would also exhibit differences in their plaque-forming cell (PFC) responses to sheep red blood cells (SRBC). They noticed that neonatally handled (H) animals exhibited lower HPA responses to a number of acute stressors in adulthood compared to nonhandled (NH) animals. They also observed that these differences also emerged as a function of chronic, intermittent cold stress. They hypothesized that H and NH animals may exhibit differences in the PFC response to SRBC under conditions of acute and/or chronic stress (H CHR and NH CHR). Exposure to acute (4 hr) cold decreased PFC responses in both H and NH animals compared to non-stressed H and NH animals. They found that decreased PFC response produced by chronic, intermittent cold stress was similar in H and NH animals and was not different from that in acutely stressed animals. In H CHR animals exposed to cold stress, the PFC response was not different from acutely stressed or chronically stressed H and NH animals. They also observed that, the PFC response in NH CHR animals exposed to cold stress was lower than all other groups studied. Thus, neonatal handling prevented prior chronic stress-induced suppression of the PFC response to a subsequent stress. They suggested that there might be subpopulations of individuals in whom prior chronic stress did not exacerbate the immune suppression produced by acute stress.

Komori *et al.* (1996) investigated the effects of one and three day (16 h/day) physically restrained or fasting stress on immunological and endocrine responses in CBF1 mice. They evaluated the influence of stressors on these responses using anti-sheep red blood cell plaque-forming assay, and by examining T cell subsets, thymus weight and endocrine hormone levels. They revealed that a significant elevation of the plaque-forming cells (PFC) was found in spleen cells in one day restrained mice, that the PFC were conversely

suppressed following three day physically restrained stress, and that the PFC were not affected by one or three day fasting stress and serum levels of norepinephrine were found to be significantly increased only in one day physically restrained mice. No change of T cell subsets and thymus weight was found in one day physically restrained mice. They concluded that the immune function was differentially affected by the duration and types of stressors.

Dabbert *et al.* (1997) studied the effects of acute thermal stress on the immune system of the northern bobwhite (*Colinus virginianus*). They noticed that neither heat stress (cycled from 30.8 to 39.0 °C over 24h) and cold stress (cycled from 3.6 to ~ 20 °C over 24h) for four consecutive days had an influence on spleen mass or measures of cell mediated and humoral immunity. They noted that the heat and cold stress had no significant effect on microhaemagglutination and bacterial agglutination titres.

Fujiwara *et al.* (1999) studied the effects stress on the induction of anti-sheep red blood cells (SRBC) and of plaque-forming cells (PFC), and on thymus weight, in BALB/c mice *in-vivo* and *in-vitro*. The efficacy of high-pressure stress in suppressing PFC and thymic involution was maximum when the stress was applied 1 h /day for 2 days before immunization with SRBC.

Fernandas (2000) investigated the neuroendocrine-immune interaction in rats challenged with sheep red blood cells (SRBC). For evaluating the immunological response were the direct plaque-forming cells (PFC) assay was used. Normal rats were injected intraperitoneally with saline or SRBC and were killed 0, 3, 4, 5, and 6 days later. They recorded the body and gland weights and serum levels of corticosterone and prolactin by radioimmunoassay. The hormone levels and gland weights of the saline conspecifics and SRBC-treated rats were found to be similar. Corticosterone and prolactin levels were significantly lower on the fifth and sixth days, respectively. The SRBC-treated rats showed a peak direct immune response on the fourth and fifth days and showed peak

corticosterone levels on the fifth day after treatment. They concluded that the former animals were under stress.

Lin *et al.* (2002) evaluated the effect of Vitamin A supplementation (9000 IU/kg), on laying performance and immune functions of heat stressed hens. They found that Vit. A supplementation had significant effect on New Castle disease virus antibody titre and ANAE (acid alpha-naphthyl acetate esterase) positive cells proportion in birds under heat stress.

Materials and Methods

3. MATERIALS AND METHODS

3.1. BIRDS

A total of 110 male day old egg type chicks (*Gallus domesticus*, local strain: Gramapriya) were procured from Kerala Agricultural University Poultry Farm, Mannuthy and reared under standard managemental conditions in a battery brooder. They were fed with commercial starter feed for the first four weeks of life. At the end of brooding period, chicks were fed with commercial adult layer mash until they attained 1 kg body weight (bw) by 3-4 months. Weekly body weight gain of all birds was recorded from the day of hatch until the completion of the experiment.

3.2. DRUG PREPARATION

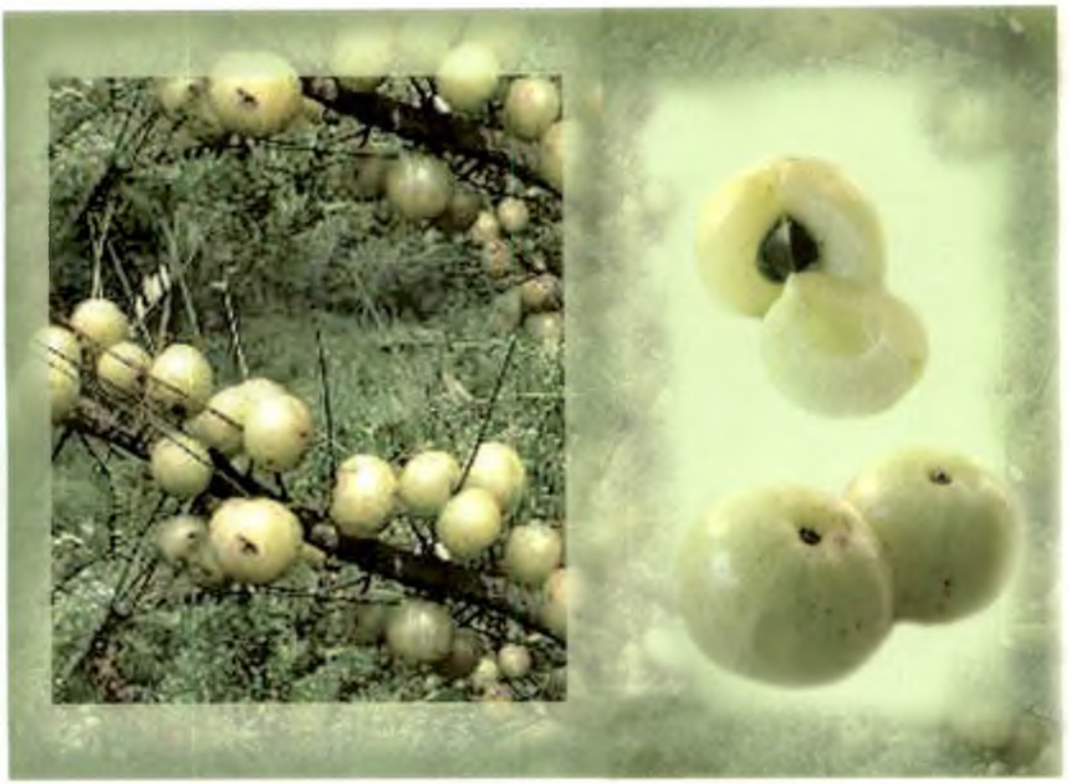
Fresh fruits of Gooseberry (GB, *Emblica officinalis*) and Indian gallnut (IGN, *Terminalia chebula*) procured from the local market were used for preparation of drug (Plate.1). After thorough washing, the seeds from the fruits were removed. The pulp of fruits was dried under shade for 2 weeks and were mixed at a ratio of 3:1 (GB:IGN) and was roasted at 60°C along with sufficient quantity of ghee, sesame oil and sugar to get a paste constituting 75 percent fruit pulp. This drug was then stored at room temperature and used for administration.

3.2.1. Administration of the drug

A toxicity trial was conducted on adult cockerels above 1kg bw by the oral administration of the drug at various dose levels of 0.50, 1.50, 2.0, 3.0, 4.50 and 6.0 g/kg bw, daily for 20 days. No significant weight loss or gain was observed reflecting the effect of drug administration on the well being of birds. Even at 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 GB-IGN combination as a non-toxic herbal preparation.

Khandelwal *et al.* (2002) and Lee *et al.* (2005) conducted studies on GB

A



B

Plate.1. A. Fruits of Gooseberry (GB, *Emblica officinalis*)
B. Fruits of Indian gallnut (IGN, *Terminalia chebula*)

and IGN and recommended combined dose of 2.0 g/kg bw in rats. Hence, for further experiments, a dose of 2.0g/kg was selected. The required quantity of GB + IBN was made into suspension using warm water (5 parts) and was mixed along with 30g of poultry feed ($^1/_3^{rd}$ of total daily ration) and fed to the experimental birds as the first meal of the day.

3.3. HEAT STRESS REGIME

A preliminary trial was carried out to assess the impact of heat exposure of 40 ±1° C for 30 min with an interval of 30 min, over a period of 3 consecutive h for 12 days. Since the mortality rate was high due to severe heat stress, a modified regime was adopted as follows.

Cockerels were exposed to intermittent heating for 60 min with an interval of 15 min, over a period of eight consecutive hours (4h/day) in a controlled environmental chamber (floor space 875 cm^2 /bird) with temperature of 40 ±1° C and relative humidity (RH) 60-70 percent for 10 days.

3.4. COOL STRESS REGIME

Cockerels were exposed to cooling for 4 h/day in an environmentally controlled chamber with temperature of 8 ±1° C and RH 40-50 per cent for 10 days.

During the period of heat/cold exposure, birds were provided with neither drinking water nor feed, inside the chamber.

3.5. EXPERIMENTAL DESIGN

A total of 80 birds were divided into 10 groups comprising 8 birds/group were utilized for the study.

Group I: Untreated and non-heat stressed (NHST) cockerels reared randomly under ambient temperature of 30±1° C and RH 65 percent for 20 days.

Group II: Drug treated (GB-IGN combination @ 2.0 g/kg bw p.o) and NHST cockerels were reared under similar conditions of temperature and humidity as Group I for 20 days.

Group III: Untreated, heat stressed (HST) at 40 ±1° C and RH 60-70 percent for 4 h/day for 5 days.

Group IV: HST at 40 ±1° C and RH 60-70 percent for 4 h/day for 5 days and drug treated (GB -IGN combination @ 2.0 g/kg bw p.o) for 20 days. The drug was administered 15 days prior to and 5 days during the experimental period of heat exposure.

Group V: Untreated, heat stressed (HST) at 40 ±1° C and RH 60-70 percent for 4 h/day for 10 days.

Group VI: HST at 40 ±1° C and RH 60-70 percent for 4 h/day for 5 days and drug treated (GB - IGN combination @ 2.0 g/kg bw p.o) for 20 days. The drug was administered 10 days prior to and 10 days during the experimental period of heat exposure.

Group VII: Untreated, cold stressed (CST) at 8 ±1° C and RH 40-50 percent for 4 h/day for 5 days.

Group VIII: CST at 8 ±1° C and RH 40-50 percent for 4 h/day for 5 days and drug treated (GB - IGN combination @ 2.0 g/kg bw p.o) for 20 days. The drug was administered 15 days prior to and 5 days during the experimental period of cold exposure.

Group IX: Cold stressed (CST) at 8 ±1° C and RH 40-50 percent for 4 h/day for 10 days.

Group X: CST at 8 ±1° C and RH 40-50 percent for 4 h/day for 5 days and drug treated (GB - IGN combination @ 2.0 g/kg bw p.o) for 20 days. The

drug was administered 10 days prior to and 10 days during the experimental period of cold exposure.

Every day rectal temperature of experimental birds was taken before keeping in the environmental chamber as well as immediately after 4 h of heat/cold exposure. The rectal temperatures of control birds, the normal room temperature and RH were recorded daily. The initial and final body weight 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. BLOOD COLLECTION

For haematological, biochemical and hormonal tests blood samples (5 ml) were collected from wing vein of birds with or with out anticoagulant (heparin) from Group III, V, VII and IX after 5 days of HST/CST, and from birds of Group IV, VI, VIII and X after 10 days of HST/CST. Blood collection was done on day 5 and 10 from all birds of Group I and II controls. For the estimation of C-reactive protein (CRP), an acute phase protein, a sample volume of 0.5 ml blood was collected from a bird in each group during heat/cold exposure. Immediately after collection, blood was incubated at 37°C for 1 ½ h and centrifuged at 3000 rpm for 20 min for better yield of serum. The serum was separated, aliquoted and stored in a deep freezer (-20°C) for further analysis. On the day of final blood collection, birds were sacrificed by cervical dislocation; spleen and right adrenals were excised out and thoroughly washed in ice cold saline (0.9%) and weighed.

3.7. TISSUE COLLECTION

Spleen and adrenals were excised out from the carcass and thoroughly washed in ice-cold saline (0.90 %) and weighed. Representative samples of adrenal gland obtained from the birds were fixed in Zenker's fluid.

The composition of Zenker's fluid:

Mercuric chloride - 50 g

Potassium dichromate - 25 g

Sodium sulphate - 10 g

Distilled water - 1000 ml

Before use, added 5 ml glacial acetic acid to 100 ml Zenker's fluid.

3.7.1. Histological procedures

The tissues were processed by routine paraffin embedding technique (Sheehan and Hrapshack, 1980). Sections were cut at four micron thickness and stained with routine Haematoxylin and Eosin stain (Bancroft and Cook, 1984) for histological studies. The stained sections were examined in detail under light microscope (400 x) and the lesions were studied.

3.8. ESTIMATION OF HAEMATOLOGICAL PARAMETERS

The hemoglobin level of blood, and differential leucocyte counts (DLC) of blood (using Leishman's stain) were determined by following standard procedures (Sastri, 1998). The method described by Natt and Herrick's (1952) was followed for total leucocyte count (TLC) and the packed cell volume (PCV) was estimated by microhematocrit method. From the TLC and DLC, absolute lymphocyte count was derived by multiplying TLC with per cent lymphocyte count.

The composition of reagent and stain used:

Natt and Herrick's fluid:

Sodium chloride - 3.88 g

Potassium chloride - 2.50 g

Disodium hydrogen phosphate, dodecahydrate - 1.44 g

Potassium dihydrogen phosphate - 0.25 g

Formalin (37per cent) - 7.50 ml

Methyl violet 2B - 0.10 g

Distilled water - 1000 ml

The above preparation was stirred overnight, filtered and used.

Leishman's stain:

Leishman's stain	- 150 mg
Methanol	- 100 ml

The above preparation was stirred overnight, filtered and used.

3.9. ESTIMATION OF BIOCHEMICAL PARAMETERS

3.9.1. Serum protein profile

3.9.1.1. *Serum total protein, albumin and globulin*

Serum total protein and albumin were estimated by Biuret method (Henry *et al.*, 1957) and Doumas method (Doumas *et al.*, 1971) respectively using Ecoline® kits (M/S. E.Merck India Limited, Mumbai). Serum globulin content was calculated as the difference between serum total protein and albumin contents.

3.9.2. Serum enzyme activities

3.9.2.1. *Estimation of Creatine kinase (CK)*

Serum creatine kinase (CK) was estimated by NAD coupled glucose-6-PO_4 oxidation method (Diwitt *et al.*, 1982) using kits (Agappe Diagnostics, Mumbai, India).

3.9.2.2. *Estimation of Lactate dehydrogenase (LDH)*

Serum lactate dehydrogenase (LDH) was estimated by kinetic method (Bergmeyer, 1975) using LDH- P kits (Agappe Diagnostics, Mumbai, India).

3.9.3. Serum electrolytes level

3.9.3.1.*Estimation of sodium and potassium*

Sodium and potassium in the serum were determined by flame photometry (Mouldin *et al.*, 1996)

3.9.4. Serum C-reactive protein (CRP) level

The Avitex – C reactive protein (CRP) latex test kit (Qualigens ® Diagnostics, India) based on method suggested by Wadsworth *et al.* (1984) was used for the semi quantification of the CRP in the serum of experimental birds. A total of 80 sera samples from birds were pooled for representative sample from each group and checked for the presence of CRP. A series of double dilutions of the serum was prepared in isotonic saline (1/2, 1/4, 1/8, and 1/16). Latex reagent and the serum (100 µl) each was added and mixed. The latex glass plate was gently and evenly rocked and rotated for 2 min and examined for agglutinated particles macroscopically and then confirmed microscopically. The CRP concentration was then calculated by multiplying the dilution factor (i.e., 2,4,8 or 16) by the detection limit value. i.e., 0.6 to get the mg/dl concentration.

3.10. ESTIMATION OF SERUM HORMONE

3.10.1. Estimation of serum cortisol concentration

Concentration of serum cortisol was estimated using the Enzyme Linked Immunosorbent Assay (ELISA) based on the method described by Arakawa *et al.* (1979) using commercial kit (M/s Equipar, Saronno, Italy).

Principle:

Anti-cortisol antibodies were immobilized on micro well plates. Cortisol in the sample competes with horseradish peroxidase (HRP) labelled cortisol for binding to immobilized antibody. After washing, enzyme substrate (proteic buffer solution containing Cortisol conjugated with HRP, ready to use) was

added. The amount of Cortisol in the sample was inversely proportional to the enzyme activity. The reaction was terminated by adding stopping solution (0.3 *M* H_2SO_4). Absorbance was measured on a ELISA plate reader at 450 nm. The inter and intra assay coefficient of variation for the determination of cortisol was done and it was found to be less than 3.2 and 5.8 % respectively.

3.10.2. Estimation of serum triiodothyronine (T_3) level

Concentration of serum triiodothyronine (T_3) was estimated using the Enzyme Linked Immunosorbent Assay (ELISA) based on the method described by Skelley *et al.* (1973) using commercial kit (M/s Genix technology, Vancouver, Canada).

Principle:

In the T_3 enzyme immunoassay (EIA), a second antibody (goat anti-mouse IgG) was coated on microtitre wells. A measured amount of bird's serum, a certain amount of mouse monoclonal anti–T_3 antibody, and a constant amount of T_3 conjugated with HRP were added to the microtitre wells. During incubation, the mouse anti-T_3 antibody was bound to the second antibody on the wells, and T_3 and conjugated T_3 compete for the limited binding sites on the anti- T_3 antibody, After 60 min incubation at room temperature, the wells were washed 5 times by water to remove unbound conjugate. A solution of TMB reagent was then added and incubated for 20 min, resulting in the development of blue colour. The colour development was stopped with the addition of 1*N* hydrocholic acid (HCl), and the absorbance was measured spectrophotometrically at 450 nm. The intensity of the colour formed was proportional to the amount of enzyme present and was inversely related to the amount of unlabeled T_3 in the sample. By reference to a series of T_3 standards assayed in the same way, the concentration of T_3 in the unknown sample was then derived.

3.10.3. Estimation of serum thyroxine (T_4) level:

Concentration of serum thyroxine (T_4) was estimated using the Enzyme Linked Immunosorbent Assay (ELISA) based on the method described by Skelley *et al.* (1973) using commercial kit (M/s Genix technology, Vancouver, Canada).

Principle:

In the thyroxine (T_4) enzyme immunoassay (EIA), a certain amount of anti- T_4 antibody was coated on microtitre wells. A measured amount of bird's serum, and a constant amount of T_4 conjugated with HRP are added to the microtitre wells. During incubation, the T_4 and conjugated T_4 compete for the limited binding sites on the anti- T_4 antibody. After 60 min incubation at room temperature (RT), the wells were washed 5 times by water to remove unbound T_4 conjugate. A solution of tetra methyl benzidine (TMB) reagent was then added and incubated for 20 min, resulting in the development of blue colour. The colour development was stopped with the addition of stop solution, and the absorbance was measured spectrophotometrically at 450 nm. The intensity of the colour formed was proportional to the amount of enzyme present and was inversely related to the amount of unlabeled T_4 in the sample. By reference to a series of T_4 standards assayed in the same way, the concentration of T_4 in the unknown sample was calculated.

3.11. ELECTROPHORETIC SEPARATION OF SERUM PROTEINS (VERTICAL SLAB SDS - PAGE) FOR IDENTIFICATION OF ANY HEAT SHOCK PROTEINS (HSPs)

Sodium dodecyl sulfate polyacrylamide (SDS) vertical slab gel electrophoresis was used for determination of molecular weight of proteins under investigation.

Materials and reagents used were:

1. Vertical slab gel electrophoresis apparatus

2. Power pack (0-500 Volts).

3. Acrylamide bisacrylamide stock solution:

Acrylamide	- 30.0 g
Bisacrylamide	- 0.8 g
Distilled water	- 100 ml

Filtered the solution through Whatman No.1 filter paper and stored in brown bottle at 4°C. This solution was stable for one month.

4. Stacking gel buffer stock (Tris-HCl, pH 6.8)

Tris	- 6.0 g
1 *M* HCl	- 48.0 ml

Adjusted pH to 6.8 and made its final volume to 100 ml with water. Filtered through Whatman No.1 filter paper and stored at 4°C.

5. Resolving gel buffer stock (Tris-HCl, pH 8.8)

Tris	- 36.3 g
1 *M* HCl	- 48.0 ml

Adjusted its pH to 8.8 and then made the final volume to 100 ml. Filtered through Whatman No.1 filter paper and stored at 4^0C.

6. 1.5% (*w/v*) Ammonium persulfate (APS) in water: Prepared by dissolving 0.15 g of APS in 10ml water. This reagent was prepared fresh just before use.

7. N, N, N', N'- Tetramethyl ethylene diamine (TEMED): 0.03ml.

8. Reservoir buffer or electrode buffer (Tris-glycine, pH 8.3)

Tris	- 3.0 g
Glycine	- 14.4 g
SDS	- 1.0 g

Adjusted its pH to 8.3 and made final volume to 1 litre with distilled water.

9. Staining solution:

Coomassie brilliant blue R-250	- 1.25 g
Methanol (70%)	- 200 ml
Glacial acetic acid	- 35 ml

Made final volume to 500 ml with distilled water. Filtered to remove any undissolved material and stored at room temperature.

10. Destaining solution:

Glacial acetic acid	- 75ml
Methanol (70%)	- 50ml

Mixed the above components and added water to make its final volume to 1 litre.

11. SDS (10%, *w/v*): Dissolved 1g SDS in 10 ml of distilled water. Stored the reagent at room temperature.

12. Sample buffer 2X: The sample preparation buffer, which contained a two folds concentration of various components, was prepared as given below:

1 *M* Tris HCl, pH 6.8	-12.5 ml
SDS	- 4.0 g
β-Mercaptoethanol	- 10.0ml
Glycerol	- 20.0 ml
1% Bromophenol blue	- 4.0 ml

Added water to make final volume to 100 ml

13. Standard molecular weight marker protein: Bovine serum albumin (BSA, 66.0 kD). Dissolved 1 mg of BSA in 1 ml of the sample buffer diluted with water in the ratio of 1:1. Then 20 µl of this mixture was loaded into one of the wells.

14. Stacking and resolving gels were prepared according to the particulars given below.

Stock solution	Stacking gel (2.5%)	Resolving gel (12.5%)
	ml of the solution	
1. Acrylamide bisacrylamide (30:08)	2.50	12.50
2. Stacking gel buffer stock solution (Tris HCl, pH 6.8)	5.00	------
3. Resolving gel buffer stock solution (Tris HCl, pH 8.8)	-----	3.75
4. 10% SDS	0.20	0.30
5. 1.5% APS	1.00	1.50
6. Water	11.30	11.95
7. TEMED	0.015	0.015
Total volume	20.00	30.00

Procedure:

Sample preparation:

The electrophoretic separations under reducing and non-reducing conditions were carried out. Reducing condition was provided by mixing the serum samples with sample buffer containing β - Mercaptoethanol and heated for three minutes by keeping in a boiling water bath and cooled to room temperature before loading. Boiled the mixture for 1 min in a boiling water bath and cooled to room temperature.

Preparation of slab gels:

Whole assembly was placed in an upright position. Various components of resolving gel were mixed as indicated in the above table except for SDS, APA and TEMED. Degassed the solution for 1 min using water pump and the above remaining components of the gel were added. Mixed gently and poured the resolving gel solution into the mould in between the clamped glass plates. Overlaid distilled water on the top as gently as possible and left for 30 min for setting of the gel. When the gel was polymerized, removed the water layer and rinsed the gel surface with stacking gel buffer. Mixed the stacking gel components in the same way as described above for the resolving gel. Poured the stacking gel and immediately inserted the supplied plastic comb in the stacking gel. Care was taken that no air bubbles were entrapped. Allowed the gel to polymerize for about 20 min. Then removed the comb. Cleaned the wells by flushing with electrode buffer using a syringe. Poured reservoir buffer in the lower and upper chambers.

Electrophoresis of samples:

20 µl samples were loaded in the sample wells. Also loaded molecular weight marker protein in one of the wells. Switched 'ON' the current maintaining, it at 15 mA for initial 15 min until the samples had traveled through the stacking gel. Then increased the current to 30 mA/gel until the bromophenol blue dye reached near the bottom of the gel slab and this required 4 hours.

After the electrophoresis was completed, turned 'OFF' and disconnected the power supply and carefully removed the gel slab from in between the glass plates. Placed the gel in a trough containing staining solution for overnight. Gel was destained with destaining solution until a clear background of the gel was obtained. Recorded the distance traveled by the molecular weight marker and the various protein bands and compared.

3.12. IMMUNOLOGICAL PARAMETERS:

3.12.1. Estimation of circulating antibody titres:

3.12.1.1. *Antigens:*

In a preliminary trial the antigenesity of bovine serum albumin (BSA), sheep red blood cells (SRBC) and rat red blood cells (RRBC) were studied by challenging then in cockerels and it was found that the titre developed against mammalian erythrocytes were much higher than that of BSA. Because of the poor antigenesity of BSA encountered in chickens, for further immunological procedures, only SRBC or RRBC was employed.

The antigen used was sheep red blood cells (SRBC) collected from adult healthy sheep (Madras red breed). Whole blood (5 ml) was drawn from the donor by jugular vein puncture, diluted in an equal volume of Alsever's anticoagulant, and centrifuged at low speed (1000 rpm) to sediment the erythrocytes. The overlying plasma and suspended leucocytes were aspirated out and discarded. The pelleted blood was resuspended in physiological saline (0.90%) and centrifuged at 3000 rpm for 10 min. The washing was repeated for four times, and finally the pellets were resuspended in physiological saline and stored as stock suspension under refrigeration.

3.12.1.2. *Trypsinization of erythrocytes:*

Trypsinized erythrocytes were prepared on the day of agglutination assay. Erythrocytes were separated from the stock solution by centrifugation at 2000 rpm for 5 min. The red blood cells were washed thrice in phosphate buffered saline (PBS) (pH 7.4) and resuspended in PBS to get a suspension of 4% $^{v}/_{v}$. To 10 ml of this suspension, 1 ml of trypsin was added and the mixture was incubated at 37°C for 1 h. The erythrocytes were then washed 3 to 4 times with PBS to remove the last traces of trypsin and finally resuspended in PBS to get a standard erythrocyte suspension with a final concentration of 2 per cent.

All birds (Group 1 to X) were immunized with 1 ml of 7% SRBC i.v, (prepared from the stock solution), seven days ahead of the actual heat/cold experimental exposures. The birds of group III, IV, V and VI were exposed to heat while group VII, VIII, IX and X were subjected to cold. A volume of 2.0 ml blood from wing vein was collected from each bird on alternate days from day 5 after immunization until the end of the experiment and serum was separated, and pooled for representative sample from each group and utilized for estimating circulating antibody titres viz., hemagglutinin titre, haemolysin titre, Mercaptoethanol (ME) sensitive (IgM) and resistant antibody (IgG) titres.

The composition of the reagents used:

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 7.4 with 1 *N* HCl or NaOH.

PBS –EDTA solution.

EDTA	– 20 mg
PBS	– 100 ml

Sterilized by autoclave.

Trypsin solution

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

Sterilized by filtration.

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% citric acid, autoclaved at 10 lbs for 15 min and stored at 4° C.

3.12.2. Hemagglutinin (HA) assay:

A microtitre plate procedure was employed for finding antibody titres (Mehra and Vaidhya, 1993). The heat inactivated (56°C for 30 min) serum sample (100µl) was serially two-fold diluted with PBS in U- shaped bottomed 96 well microtitre plate (Tarson's India Ltd.). An equal volume of 2 % trypsinized erythrocyte suspension was then added to each well and incubated for 1 h at room temperature. The degree of agglutination was evaluated macroscopically and the HA titres were expressed as the $\log_2$ of the reciprocal of the highest dilutions giving visible agglutination.

3.12.3. Haemolysin (HL) response:

The haemolysin (HL) titres of heat inactivated serum samples were determined using microtitre procedure (Mehra and Vaidhya, 1993). Serum samples (100µl) were two-fold diluted with PBS. After the addition of 100µl trypsinized SRBC, 25 µl of complement (fresh serum obtained from suitable chicken donors) was added to all wells and the reaction mixture was incubated at 37°C for 30 min. Titres were determined by looking for hemolysed supernatant in each well and were expressed as the $\log_2$ value like agglutinin levels.

3.12.4. Mercaptoethanol (ME) sensitive (IgM) and resistant antibody (IgG):

Equal volumes (100 µl) of heat inactivated serum and 0.2 M Mercaptoethanol (ME) in PBS (pH 7.4) were mixed and incubated at 37°C for 30 min prior to serial dilution (Mehra and Vaidhya, 1993). Agglutination tests were conducted as mentioned in **3.12.2**, and titre was recorded as ME-resistant antibody (IgG). The reduction of titre from haemaglutinin (HA) titre due to ME treatment was recorded as ME - sensitive antibody (IgM) titre.

3.13. TISSUE CULTURE:

3.13.1. Determination of Antibody forming cells

3.13.1.1. *Antigens.*

The antigen used was rat red blood cells (RRBC), collected from adult healthy male rats (Sprague Dawely). About 3 ml blood was drawn from the donor by retro orbital plexus puncture using heparinised capillary tubes, and collected in an equal volume of Alsever's solution, and centrifuged at low speed (1000 rpm) to sediment the erythrocytes. The overlying plasma and suspended leucocytes were aspirated out and discarded. The pelleted blood was resuspended in physiological saline (0.90%) and centrifuged at 3000 rpm for 10 min. The washing was repeated four times, and finally the pellet was resuspended in physiological saline to get RRBC stock suspension.

3.13.1.2. *Experimental design*

A total of 30 cockerels were divided into 6 groups comprising 5 birds/group were utilized for the study.

Group I: Untreated and non-heat stressed (NHST) birds reared randomly under ambient temperature of 30±1° C and 65 percent relative humidity (RH).

Group II: NHST and drug treated (GB - IGN combination @ 2.0g/kg bw p.o) birds were reared under similar conditions of temperature and humidity as Group I for 20 days.

Group III: Untreated, heat stressed (HST) at 40 ±1° C and RH 60-70 percent for 4 h/day for 10 days.

Group IV: HST at 40 ±1° C and RH 60-70 percent for 4 h/day for 10 days and drug treated (GB - IGN - combination @ 2.0g/kg bw p.o) for 20 days.

The drug was administrated 10 days prior to and 10 days during the experimental period of heat exposure.

Group V: Untreated, cold stressed (CST) at 8 ±1° C and RH 40-50 percent for 4 h/day for 10 days.

Group VI: CST at 8 ±1° C and RH 40-50 percent for 4 h/day for 5 days and drug treated (GB - IGN combination @ 2.0g/kg bw p.o) for 20 days. The drug was administrated 10 days prior to and 10 days during the experimental period of cold exposure.

3.13.1.3. *Immunization.*

All birds of group II to VI were immunized with 1 ml i.v of 7 % RRBC. On the following day after immunization, birds of group III to VI were subjected to heat /cold exposure. A bird from each group (I to VI) was sacrificed by cervical dislocation on day 2,4,6,8 and 10^{th} day of heat/cold exposure, which corresponds to 3,5,7,9, and 11^{th} day after immunization.

3.13.2. Jerne's plaque asssay

The spleen was excised out, washed with cold physiological saline (0.9%), and immersed in Hank's Balanced Salt Solution (HBSS) (Hi Media laboratories, Mumbai) under sterile conditions. The capsule of the spleen was incised and removed. The parenchyma was processed as follows. A single cell suspension was prepared in HBSS by triturating spleen parenchyma in required quantity of sterile HBSS under cold conditions. The suspension was sieved and the number of viable cells was estimated by Trypan blue dye exclusion method i.e., 100 µl of spleen suspension was added to 100µl of 1 percent trypan blue and 800µl of normal saline. Mixed well and after two min, hemocytometer was loaded. The viable cells excluded the dye while non-viable cells appears blue. The stained and unstained cells were counted and the percentage of cell death was calculated. Cell viability generally exceeded 80 percent. Depending up on the viability,

spleen suspension was diluted with HBSS to get a concentration of 7.5 x 10^6 viable cells/ml.

The composition of stain used:

Trypan blue:

Trypan blue – 100 mg

Saline (0.9 %) – 100 ml

The above preparation was stirred overnight, filtered and used.

Procedure:

A volume of 500 µl of 0.50 % agarose in HBSS was taken in tubes kept at 45° C and 100 µl RRBC (15 %) in physiological saline, 300 µl of cold HBSS and 300 µl of spleen cells (≈ 2.5 million viable cells.) were added and mixed well. The contents of the tubes were poured in to grease free slide, spread into an area of 1''x 2'' and allowed to solidify. Fresh chicken serum, (1:10 diluted with PBS, pH 7.4) was used as complement source. The slides were kept upside down in an incubator rack and the space between the slides and rack was filled with complement. The slides were incubated for 1h at 37° C. In the presence of complement, antibody produced by the lymphoid cells from birds, immunized with RRBC, cause lysis of red cells in its vicinity (plaques) in a solid support in the presence of complement (Jerne and Nordin, 1963). Plaques so formed were found as empty spaces due to hemolysis, and was counted in a colony counter and later confirmed under low power light microscope (5x), and expressed not only as the number of plaques forming cells (PFC) per million spleenocytes but also as percentage PFC.

3.13.3. Rosette- forming cell (RFC) immunocytoadherence assay:

The method suggested by Dagg *et al.* (1977) was followed for RFC immunocytoadherence assay. A suspension of spleen cells was prepared as for the plaque assay. For this assay, a mixture consisting of 300µl cell suspension (≈ 2.5 million viable cells), 500 µl of 15% RRBC suspension, and 850 µl of HBSS

were prepared. The mixture was centrifuged at 2000 rpm for 1 min and the pellet was stored in the refrigerator for 30 min. It was gently resuspended with a Pasteur pipette and loaded in a hemocytometer. The number of rosettes (clusters of four or more mammalian erythrocytes adhering to a chicken lymphoid cell), spread over all the WBC counting squares was counted and the result was expressed not only as the number of rosette forming cells (RFC) per million spleenocytes but also as percentage RFC.

3.14. STATISTICAL ANALYSES OF DATA

The data obtained were statistically analyzed using one way Analysis of Variance (ANOVA) test and further analyzed by Least Significant Difference (LSD) test for significance among homogenous variances (Snedecor and Cochran, 1994).

Results

4. RESULTS

4.1. EFFECT OF HEAT STRESS ON BODY TEMPERATURE

Body temperature was found to be significantly ($P<0.05$) increased in Group III (untreated-5 days HST), IV (GB+IGN treated-5 days HST), V (untreated-10 days HST) and VI (GB+IGN treated-5 days HST) cockerels when compared to Group I (untreated-NHST) and II (GB+IGN treated-NHST) control cockerels. In HST cockerels, the mean body temperature reached 44.00 ± 0.20°C by the end of 4h exposure to heat, while control birds recorded mean rectal temperature of 42.90±0.20°C. The HST cockerels lost an average of 35.00g body weight daily upon 4h exposure to 40.00±1°C, which accounts for an average loss of 3.40% of body weight where as Group II (GB+IGN treated- NHST) cockerels did not show any change in the body weight. Cockerels exhibited diarrhea and a great deal of behavioral changes such as gular flutter (panting), drooping of wings, prostration and drowsiness during the period of heat stress.

4.2.EFFECT OF HEAT STRESS ON CERTAIN HAEMATOLOGICAL PARAMETERS

4.2.1. Haemoglobin (Hb) concentration

The mean Hb concentration for Group I (untreated-NHST) control cockerels was 10.63 ± 0.23 g per cent, which increased significantly ($P<0.05$) to 11.81 ± 0.48 g per cent in Group III (untreated-5 days HST) but there was no significant difference in Group V, (untreated-10 days HST) cockerels when compared with Group I cockerels. The mean Hb concentration of Group II (GB+IGN treated-NHST) cockerels was 10.06 ± 0.02 g per cent which increased significantly ($P<0.05$) to 11.68 ± 0.44 g per cent in Group IV (GB+IGN treated-5 days HST) cockerels, where as Group II cockerels did not show significant difference with Group VI (GB+IGN treated-10 days HST) cockerels (Table 1).

Table 1. Effect of heat stress on certain haematological parameters (Mean ± SE, n=8)

Groups	Haemoglobin (Hb) g%	Packed cell volume (PCV) %	Total leucocyte count (TLC) x103/µl	Lymphocytes (L) %	Heterophils (H) %	H/L Ratio
I	$10.63^{b} \pm 0.23$	$34.30^{bc} \pm 1.34$	$10975.00^{ab} \pm 741$	$59.63^{a} \pm 4.65$	$40.38^{d} \pm 4.65$	$0.77^{d} \pm 0.13$
II	$10.06^{bc} \pm 0.02$	$32.15^{bc} \pm 0.99$	$12550.00^{a} \pm 472$	$60.75^{a} \pm 0.75$	$39.13^{d} \pm 0.87$	$0.63^{d} \pm 0.28$
III	$11.81^{a} \pm 0.48$	$37.50^{a} \pm 1.45$	$4375.00^{e} \pm 324$	$21.25^{d} \pm 1.25$	$78.75^{a} \pm 1.25$	$3.82^{a} \pm 0.27$
IV	$11.68^{a} \pm 0.44$	$35.19^{ab} \pm 0.94$	$6000.00^{d} \pm 250$	$22.89^{cd} \pm 1.04$	$77.13^{ab} \pm 1.04$	$3.19^{b} \pm 0.13$
V	$9.75^{bc} \pm 0.19$	$34.68^{ab} \pm 0.96$	$8000.00^{c} \pm 535$	$29.13^{bc} \pm 2.49$	$70.88^{bc} \pm 2.48$	$2.86^{b} \pm 0.40$
VI	$9.63^{c} \pm 0.18$	$31.29^{c} \pm 0.68$	$10375.00^{b} \pm 498$	$35.38^{b} \pm 1.29$	$64.63^{c} \pm 1.29$	$1.86^{c} \pm 0.11$

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg bw for 10 days orally) and non- heat stressed controls.

Group III – Untreated and 5 days heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and 5 days heat stressed.

Group V – Untreated and 10 days heat stressed.

Group VI – (GB+IGN) treated and 10 days heat stressed.

4.2.2. Packed cell volume (PCV)

The mean value of PCV in Group III (untreated-5 days HST) cockerels was 37.50 ± 1.45 per cent and it was significantly (P<0.05) higher than that in Group I (untreated-NHST) cockerels (34.30± 1.34 per cent), later on decreased non significantly to reach 34.68± 0.96 per cent in Group V (untreated-10 days HST) cockerels. Group II (GB+IGN treated-NHST) cockerels showed a mean PCV value of 32.15 ± 0.99 per cent, which showed no significant difference with Group IV (GB+IGN treated-5 days HST) where as the PCV value decreased significantly to 31.29 ± 0.68 per cent in Group VI (GB+IGN treated-10 days HST) cockerels (Table 1).

4.2.3. Total leucocyte (WBC) count

The mean total leucocyte count of Group I (untreated-NHST) cockerels was 10975.00 ± 741/µl and it was significantly (P<0.05) higher than Group III (untreated-5 days HST) cockerels and Group V (untreated-10 days HST) cockerels which showed a mean total leucocyte count of 4375.00± 324 /µl and 8000.00 ±535 /µl respectively. Group II (GB+IGN treated-NHST) cockerels which exhibited WBC count of 12550.00 ± 472/µl differed significantly (P<0.05) with Group IV (GB+IGN treated- 5 days HST) and Group VI (GB+IGN treated-10 days HST) cockerels which showed an average count of 6000.00 ± 250 /µl and 10375.00 ± 498 /µl respectively (Table 1).

4.2.4. Lymphocyte (L) count

Group I (untreated-NHST) cockerels had significantly (P<0.05) higher mean lymphocyte count of 59.63 ± 4.65 per cent than those exhibited by Group III (untreated-5 days HST) and Group V (untreated-10 days HST) which showed the values as 21.25±1.25 and 29.13±2.49 per cent respectively. The mean lymphocyte count of Group II (GB+IGN treated-NHST) cockerels was 60.75± 0.75 per cent which decreased significantly (P<0.05) to 22.89 ± 1.04 in Group IV

(GB+IGN treated- 5 days HST) and to 35.38± 1.29 per cent in Group VI (GB+IGN treated 10 days HST) cockerels (Table 1).

4.2.5. Heterophil (H) count

The mean heterophil count for Group I (untreated and NHST) cockerels was 40.38± 4.65 per cent which increased significantly ($P<0.05$) to 78.75± 1.25 and 70.88 ± 2.48 per cent in Group III and Group V cockerels respectively. Similarly, there was a significant ($P<0.05$) increase in mean heterophil count of Group IV (77.13± 1.04 per cent) and Group VI (64.63 ± 1.29 per cent) when compared to Group II (39.13± 0.87 per cent) cockerels (Table 1).

4.2.6. Heterophil/ Lymphocyte (H/L) ratio

The mean H/L ratio in Group I (untreated-NHST) cockerels was0.77 ± 0.13, which increased significantly ($P<0.05$) to 3.82 ± 0.27 in Group III (untreated-5 days HST) and 2.86 ± 0.40 in Group V (untreated-10 days HST) cockerels. The mean H/L ratio in Group II (GB+IGN treated-NHST) cockerels was 0.63 ± 0.28, which increased significantly ($P<0.05$) to 3.19 ± 0.13 and 1.86 ± 0.11 in Group IV and Group VI cockerels respectively (Table 1).

4.3. EFFECT OF HEAT STRESS ON BIOCHEMICAL PARAMETERS

4.3.1. Serum protein profile:

4.3.1.1. *Serum total protein content*

The mean serum total protein content of the birds in different groups did not differ significantly ($P>0.05$) either by GB+IGN treatment, heat stress or by both. The values ranged from 4.75 ± 0.16 g/dl in Group III (untreated-5 days HST) to 5.75 ± 0.67 g/dl in Group VI (GB+ IGN treated-10days HST) cockerels (Table 2).

Table 2.Effect of heat stress on serum protein profile (Mean ± SE, n=8)

Groups	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
I	$5.38^{a} \pm 0.53$	$2.71^{a} \pm 0.22$	$2.09^{a} \pm 0.13$
II	$5.50^{a} \pm 0.27$	$3.40^{a} \pm 0.17$	$2.54^{a} \pm 0.43$
III	$4.75^{a} \pm 0.16$	$2.66^{a} \pm 0.39$	$2.34^{a} \pm 0.25$
IV	$5.00^{a} \pm 0.63$	$2.70^{a} \pm 0.12$	$2.05^{a} \pm 0.09$
V	$5.13^{a} \pm 0.29$	$3.14^{a} \pm 0.16$	$1.99^{a} \pm 0.14$
VI	$5.75^{a} \pm 0.67$	$3.45^{a} \pm 0.46$	$2.30^{a} \pm 0.23$

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg bw for 10 days orally) and non- heat stressed controls.

Group III – Untreated and 5 days heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and 5 days heat stressed.

Group V – Untreated and 10 days heat stressed.

Group VI – (GB+IGN) treated and 10 days heat stressed.

4.3.1.2. *Albumin content*

There was no significant (P>0.05) difference in the mean serum albumin content of cockerels between different groups. The values ranged from 2.66 ± 0.39 g/dl in Group III (untreated- 5 days HST) to 3.45 ± 0.46 g/dl in Group VI (GB+IGN treated-10 days HST) cockerels (Table 2).

4.3.1.3. *Globulin content*

The mean serum globulin content of all the birds in different groups did not differ significantly (P>0.05) either by GB+IGN treatment and heat stress or by both. The values ranged from 1.99 ± 0.14 g/dl in Group V (un treated-10 days HST) to 2.54 ± 0.43 g/dl in Group II (GB+IGN treated-NHST) cockerels (Table 2).

4.3.2. Serum enzyme activities

4.3.2.1. *Serum lactate dehydrogenase (LDH) activity*

The mean LDH activity in Group I (untreated-NHST) cockerels was 1374.75 ± 4.02 U/l. A significant (P<0.05) increase to 2057.25 ± 63.39 U/l in Group III (untreated-5 days HST) cockerels and 2125.75 ± 14.21 U/l in Group V (untreated-10 days HST) cockerels was also observed. In Group II (GB+IGN treated-NHST) cockerels the LDH activity was 1082.13 ± 11.47 U/l, which did not show significant difference with Group IV (GB+IGN treated-5 days HST) cockerels. However, there was a significant (P<0.05) increase to 2123.25 ± 21.57 U/l in Group VI (GB+IGN treated-10 days HST) cockerels (Table 3).

4.3.2.2. *Serum Creatine kinase (CK) activity*

The mean serum CK activity in Group I (untreated-NHST) cockerels was 1583.88 ± 5.49 U/l which increased significantly (P<0.05) to 2100.53± 61.05 U/l in Group III (untreated-5 days HST) cockerels. Further significant (P<0.05) elevation to 3465.88 ± 13.37 U/l was observed in Group V (untreated-10 days

Table 3. Effect of heat stress on certain serum enzyme and electrolyte levels (Mean ± SE, n=8)

Groups	Lactate dehydrogenase (LDH) U/l	Creatine kinase (CK) U/l	Serum Sodium (mEq/L)	Serum Potassium (mEq/L)
I	1374.75^{b}±4.02	1268.25^{f}±6.47	133.55^{a} ± 5.03	4.64^{ab}±0.12
II	1082.13^{c}±11.47	1583.88^{d}±5.49	146.34^{a}± 7.39	5.03^{a}±0.18
III	2057.25^{a}±63.39	2100.53^{b}±61.05	143.46^{a}± 1.69	4.76^{ab}±0.19
IV	1125.25^{c}±21.34	1428.88^{e}±89.32	142.75^{a}± 3.38	3.94^{c}±0.23
V	2125.75^{a}±14.21	3465.88^{a}±13.37	140.81^{a}± 4.51	3.91^{c}±0.06
VI	2123.25^{a}±21.57	1714.00^{c}±13.68	132.74^{a}± 5.50	4.31^{bc}±0.22

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg bw for 10 days orally) and non- heat stressed controls.

Group III – Untreated and 5 days heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and 5 days heat stressed.

Group V – Untreated and 10 days heat stressed.

Group VI – (GB+IGN) treated and 10 days heat stressed.

HST) cockerels. The mean serum CK activity in Group II (GB+IGN treated-NHST) cockerels was 1268.25 ± 6.47 U/l which increased significantly (P<0.05) to 1428.88± 89.32 U/l in Group IV (GB+IGN treated-5 days HST) cockerels and further increased to 1714.00 ± 13.68 U/l in Group VI (GB+IGN treated 10 days) HST cockerels (Table3).

4.3.3. Serum electrolytes level

4.3.3.1. *Serum sodium level*

There was no significant (P>0.05) difference in the mean serum sodium content of cockerels in different groups. The values ranged from 132.74± 5.50 mEq/l in Group VI (GB+ IGN treated-10 days HST) to 146.34 ± 7.39 mEq/l in Group II (GB+IGN treated-NHST) cockerels (Table 3).

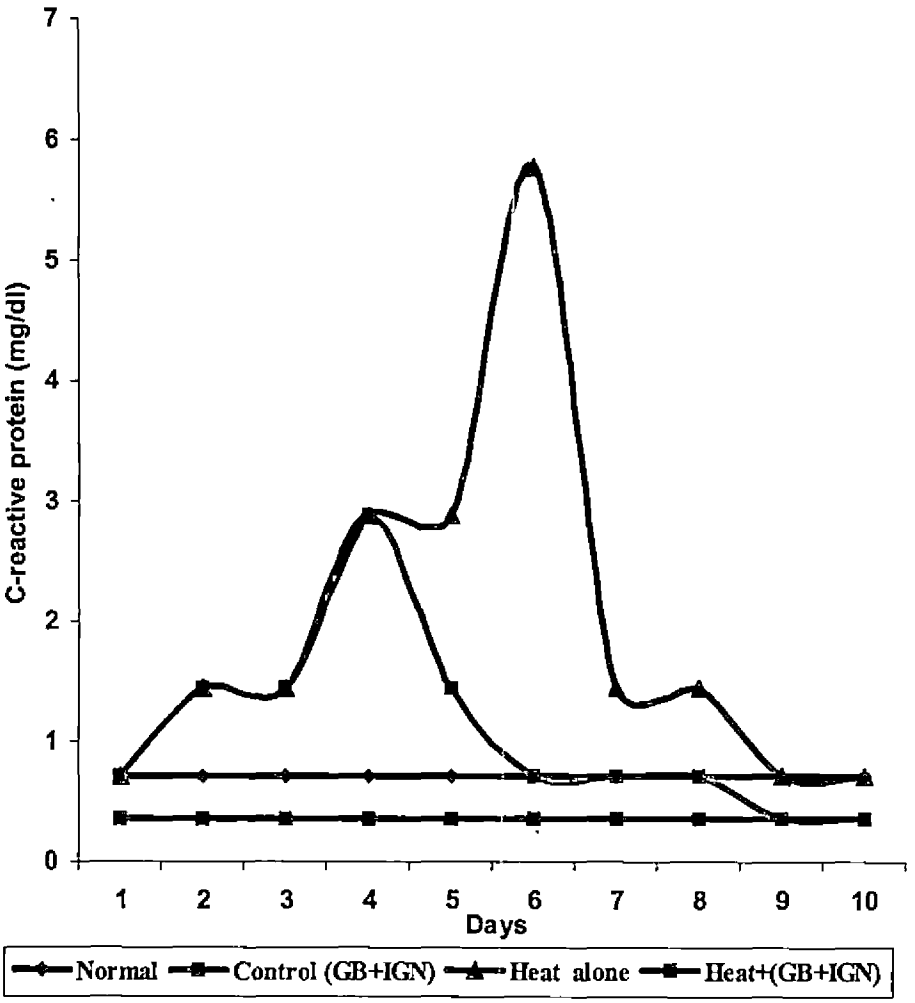
4.3.3.2. *Serum potassium level*

The mean serum potassium level in Group I (untreated-NHST) cockerels was 4.64 ± 0.12 mEq/l and it non significantly increased to 4.76± 0.19 mEq/l in Group III (untreated-5 days HST) cockerels, and later on, in Group V (untreated-10 days HST) cockerels the K level decreased significantly to reach 3.91 ± 0.06 mEq/l. The mean serum potassium level in Group II (GB+IGN treated-NHST) cockerels was 5.03 ± 0.18 mEq/l that significantly (P<0.05) decreased to 3.94 ± 0.23 mEq/l in Group IV (GB+IGN treated-5 days HST) cockerels. While, Group VI (GB+IGN treated-10 days HST) cockerels with a mean value of 4.31± 0.22mEq/l showed a non-significant increase to reach the basal line (Table 3).

4.3.6. Serum C-reactive protein (CRP) level

The effect of heat stress on serum CRP level in normal and GB+IGN treated cockerels are shown in Fig.1. Both the Group I (untreated-NHST) and Group II (GB+IGN treated-NHST) cockerels maintained a stable value of 0.72 and 0.36 mg/dl respectively throughout the experiment. However,

Fig.8.Effect of heat stress on serum C-reactive protein (CRP) level



Group III and V (untreated-5 and 10 days HST) cockerels as well as Group IV and VI (GB+IGN treated-5 and 10 days HST) cockerels exhibited a value of 1.44 mg/dl on 2nd day of heat exposure, which was four times higher than Group II and two times higher than the Group I cockerels. In Group III and V (untreated-5 and 10 days HST) cockerels, a peak value of 5.76 mg/dl was observed on 6th day of heat exposure and thereafter the value declined. Cockerels of Group IV and VI (GB+IGN treated-5 and 10 days HST) exhibited a value of 1.44mg/dl on 2nd and 3rd day of heat exposure, which then increased to attain a peak level of 2.88 mg/dl on 4th day of heat exposure and then decreased to gradually to 0.36 mg/dl, which persisted for 4 more days.

4.4. EFFECT OF HEAT STRESS ON CERTAIN SERUM HORMONES LEVEL

4.4.1. Serum cortisol concentration

The mean serum cortisol concentration showed a significant ($P<0.05$) increase from 32.00 ± 0.71 ng/dl in Group I (untreated-NHST) cockerels to 46.00 ± 0.43 ng/dl in Group III (untreated-5 days HST) cockerels. The Group V (untreated-10 days HST) cockerels also showed a significant ($P<0.05$) increase to 42.00 ± 0.71 ng/dl when compared with Group I cockerels. The mean serum cortisol concentration in Group II (GB+IGN treated-NHST) cockerels was 27.50 ± 0.52 ng/dl which increased significantly ($P<0.05$) to 43.63± 0.49 ng/dl in Group IV (GB+IGN treated-10 days HST) cockerels and later on significantly ($P<0.05$) decreased to 35.50 ± 0.44 ng/dl in Group VI (GB+IGN treated-10 days HST) cockerels (Table 4).

4.4.2. Serum triiodothyronine (T_3) concentration

The mean serum triiodothyronine (T_3) concentration in Group I (untreated-NHST) cockerels was 106.00 ± 1.93 ng/dl which increased significantly ($P<0.05$) to 160.00 ± 8.71 ng/dl in Group III (untreated-5 days HST) cockerels and remained at significantly ($P<0.05$) higher level of 147.00 ± 1.66 ng/dl in Group V (untreated-10 days HST) cockerels when compared with Group I cockerels. In

Table 4.Effect of heat stress on serum hormone profile (Mean ± SE, n=8)

Groups	Serum Cortisol (ng/ml)	Serum T_3 (ng/dl)	Serum T_4 (µg/dl)
I	32.00^{e}±0.71	106.00^{c}±1.93	3.55^{a}±0.24
II	27.50^{f}±0.52	44.25^{d}±1.03	3.49^{ab}±0.29
III	46.00^{a}±0.43	160.00^{a}±8.71	4.00^{a}±0.13
IV	43.63^{b}±0.49	136.25^{b}±9.98	2.86^{c}±0.09
V	42.00^{c}±0.71	147.00^{a}±1.66	3.36^{bc}±0.06
VI	35.50^{d}±0.44	116.25^{c}±3.24	3.38^{bc}±0.16

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg.bw for 10 days orally) and non- heat stressed controls.

Group III – Untreated and 5 days heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and 5 days heat stressed.

Group V – Untreated and 10 days heat stressed.

Group VI – (GB+IGN) treated and 10 days heat stressed.

Group II (GB+IGN treated-NHST) cockerels the T_3 level was 44.25 ± 1.03 ng/dl which increased significantly ($P<0.05$) to 136.25 ± 9.98 ng/dl in Group IV (GB+IGN treated-5 days HST) cockerels, which decreased significantly ($P<0.05$) to 116.25 ± 3.24 ng/dl in Group VI (GB+IGN treated-10 days HST) cockerels (Table 4).

4.4.3. Serum thyroxine (T_4) concentration

The mean serum thyroxine (T_4) concentration increased non significantly ($P>0.05$) to 4.00 ± 0.13 µg/dl in Group III (untreated-5 days HST) cockerels when compared to 3.55 ± 0.24 µg/dl exhibited by Group I (untreated-NHST) cockerels which then decreased significantly ($P<0.05$) to 3.38± 0.16µg/dl in Group V (untreated-10 days HST) cockerels. Group II (GB+IGN treated-NHST) cockerels exhibited mean serum T_4 level of 3.49 ± 0.29 µg/dl, which decreased significantly ($P<0.05$) to 2.86 ± 0.09 µg/dl in Group IV (GB+IGN treated-5 days HST) and then increased non significantly ($P>0.05$) to 3.36 ± 0.06 µg/dl in Group VI (GB+IGN treated-10 days HST) cockerels when compared with the Group II cockerels (Table 4).

4.4. ELECTROPHORETIC SEPARATION OF SERUM PROTEINS (VERTICAL SLAB SDS - PAGE)

The electrophoretic separation of serum proteins of chicken under non-reducing conditions did not show any appreciable changes in any of the groups tested (Plate 2). The electrophoretic separation of serum proteins carried out under reducing conditions revealed that in Group III (GB+IGN treated-5days HST) and Group II (GB+IGN treated-NHST) cockerels (lane 5 and 6), appearance of newer protein bands with low molecular weight (lesser than that of bovine serum albumin) could be noticed while some high molecular protein bands were found missing (Plate 3).

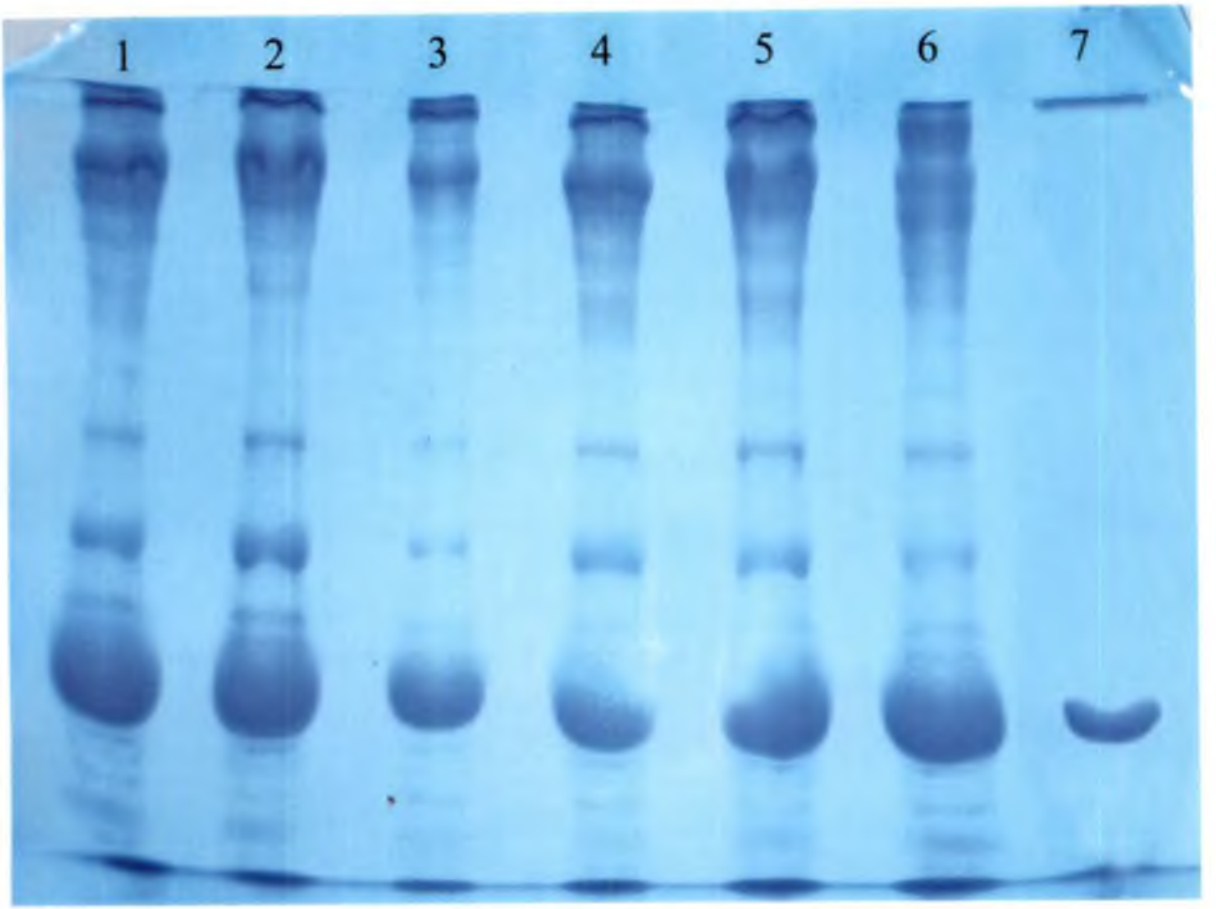


Plate 2. SDS PAGE of serum proteins
(under non-reducing conditions)

Lane 1 : Serum of (GB+IGN) treated and 10 days heat stressed cockerels.

Lane 2 : Serum of 10 days heat stressed cockerels.

Lane 3 : Serum of (GB+IGN) treated and 5 days heat stressed cockerels.

Lane 4 : Serum of 5 days heat stressed cockerels.

Lane 5 : Serum of (GB+IGN) treated and non heat stressed cockerels.

Lane 6 : Serum of untreated and non heat stressed cockerels.

Lane 7 : Bovine serum albumin cockerels.

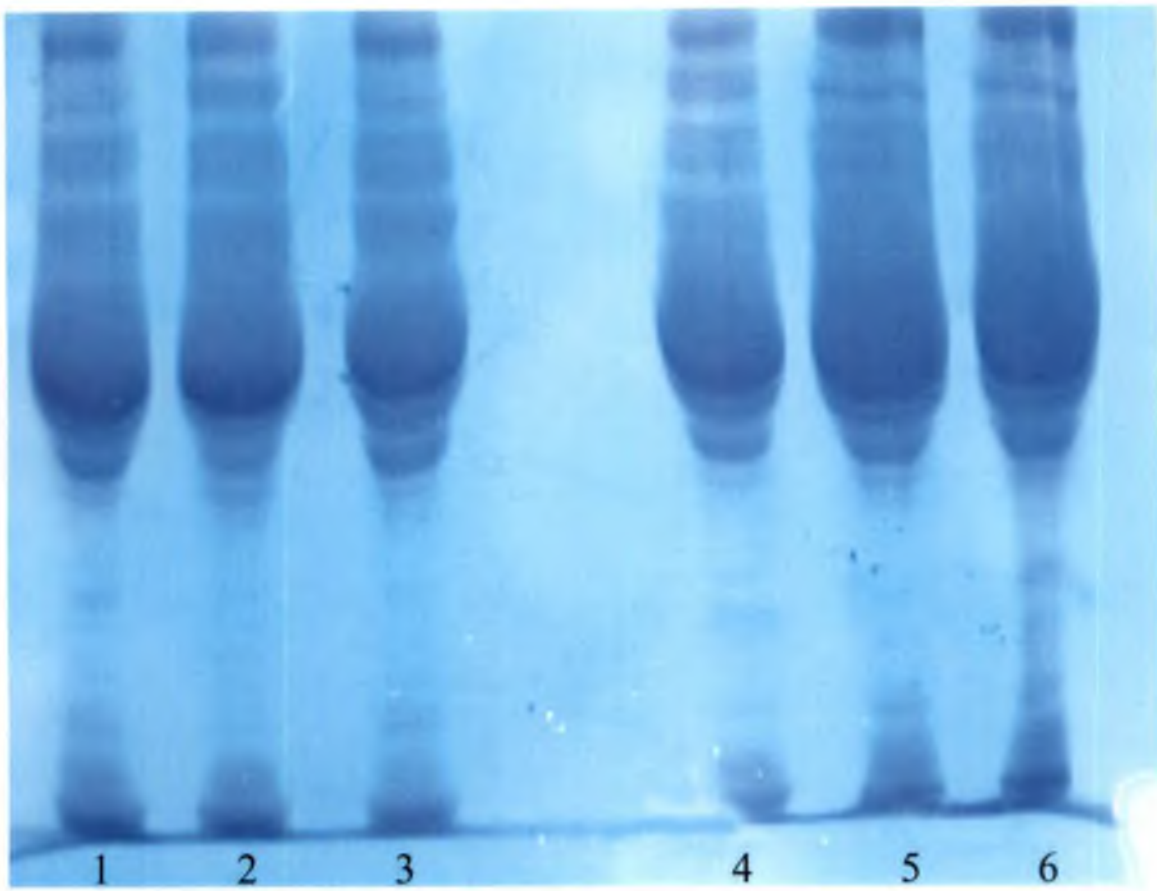


Plate 3. SDS PAGE of serum proteins
(under reducing conditions)

Lane 1 : Serum of untreated and non heat stressed cockerels.

Lane 2 : Serum of (GB+IGN) treated and non heat stressed cockerels.

Lane 3 : Serum of (GB+IGN) treated and 5 days heat stressed cockerels.

Lane 4 : Serum of (GB+IGN) treated and 10 days heat stressed cockerels.

Lane 5 : Serum of 5 days heat stressed cockerels.

Lane 6 : Serum of 10 days heat stressed cockerels.

4.5. EFFECT OF HEAT STRESS ON IMMUNE RESPONSES

4.5.1. Haemaglutinin (HA) assay

The effect of heat stress on serum anti-sheep red blood cell (anti-SRBC) haemagglutinin (HA) titre in normal and GB+IGN treated cockerels was shown in Fig.2. The 5th day after immunization corresponds to first day of heat exposure. The $\log_2$ HA titre was found to be 3 for Group I (untreated-NHST i.e., normal) and Group III and V (untreated-5 and 10 days HST i.e., heat alone) cockerels on 5th day after immunization, while a $\log_2$ titre value of 7 was exhibited by Group II (GB+IGN treated-NHST i.e., control (GB+IGN)) and Group V and IV (GB+IGN treated-5 and 10 days HST i.e., heat + (GB+IGN)) cockerels. On 11th day after immunization the peak $\log_2$ titre values of 10 and 11 was observed in normal and control (GB+IGN) cockerels respectively. Thereafter the $\log_2$ titre values decreased to 6 in normal and 7 in control (GB+IGN) cockerels and persisted till the end of the experiment. While in untreated-HST cockerels (heat alone) the peak $\log_2$ titre value of 8 was observed on day 8 which declined to $\log_2$ titre of 5 for next 5 days and finally reached a $\log_2$ titre of 4 by 14th day after immunization. However, heat + (GB+IGN) cockerels a peak $\log_2$ titre value of 10 was encountered on day 8 which then immediately declined to a $\log_2$ titre value of 7 and it was maintained till the end of the experiment.

4.5.2. Haemolysin (HL) response

The effect of heat stress on serum anti-sheep red blood cell (anti-SRBC) haemolysin (HL) titre in normal and GB+IGN treated cockerels was shown in Fig.3. The 5th day after immunization corresponds to first day of heat exposure. The $\log_2$ HL titre was found to be 2 for Group I (untreated-NHST i.e., normal) and Group III and V (untreated-5 and 10 days HST i.e., heat alone) cockerels on 5th day after immunization, while a $\log_2$ titre value of 6 was exhibited by Group II (GB+IGN treated-NHST i.e., control (GB+IGN)) and Group V and IV (GB+IGN treated-HST i.e., heat + (GB+IGN)) cockerels. On 11th day after immunization

Fig.2. Effect of heat stress on haemagglutinin (HA) titre

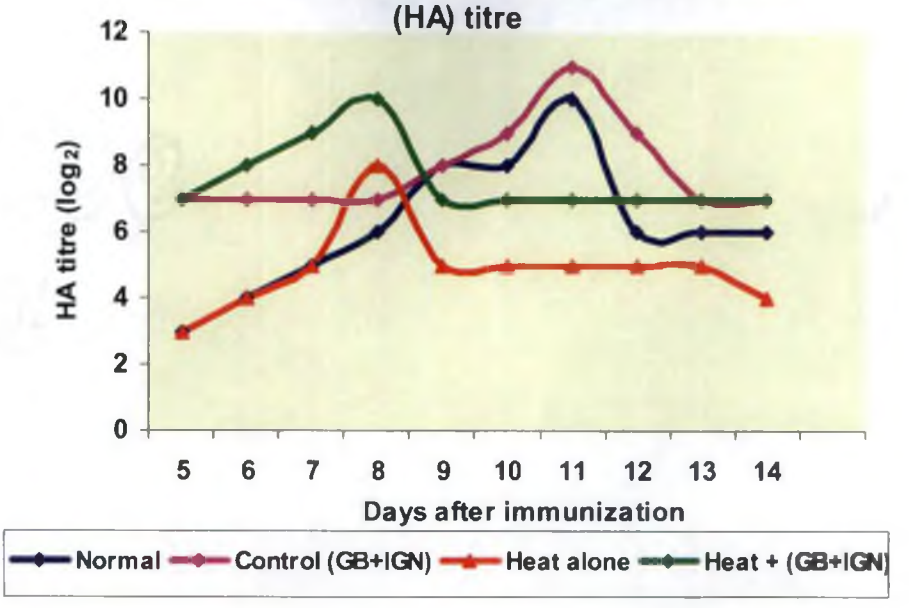
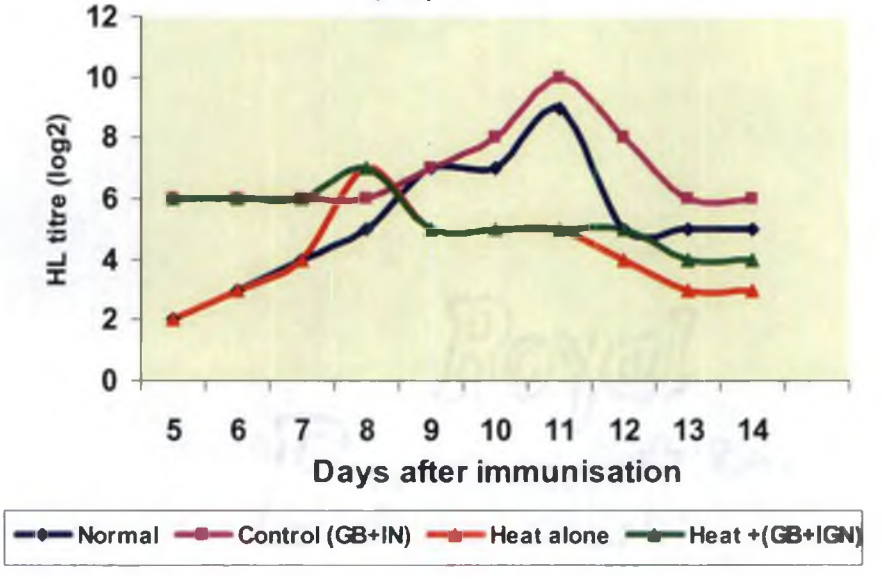


Fig.3.Effect of heat stress on haemolysin (HL) titre



the peak log_2 titre values of 9 and 10 was observed in normal and control (GB+IGN)cockerels respectively. Thereafter the log_2 titre values decreased to 5 in normal and 6 in control (GB+IGN) cockerels and persisted till the end of the experiment. While in untreated-HST cockerels (heat alone) the peak log_2 titre value of 7 was observed on day 8 which declined to log_2 titre of 5 for next 3 days and finally reached a log_2 titre of 3 by 14^{th} day after immunization. However, heat + (GB+IGN) cockerels a peak log_2 titre value of 7 was encountered on day 8 which then immediately declined to a log_2 titre value of 5 for next 4 days and finally reached a log_2 titre of 4 and it was maintained till the end of the experiment.

4.5.3. Serum mercaptoethanol resistant antibody (Ig G) level

The effect of heat stress on anti-sheep red blood cell (anti-SRBC) serum IgG titre in normal and GB+IGN treated cockerels was shown in Fig.4. The 5^{th} after immunization corresponds to first day of heat exposure. The log_2 IgG titres of Group I (untreated-NHST i.e., normal) and Group III and V (untreated-5 and 10 days HST i.e., heat alone) cockerels on 5^{th} day after immunization was 2, while a log_2 titre value of 5 was exhibited by Group II (GB+IGN treated-NHST i.e., control (GB+IGN)) and Group V and IV (GB+IGN treated-5 and 10 days HST i.e., heat + (GB+IGN)) cockerels. In normal cockerels, peak log_2 titre value of 5 was observed on 9^{th} day and that was maintained till day 11 and then it declined to a value of 4 till the end of the experiment. In untreated-HST (heat alone) cockerels the highest log_2 titre of 5 was reached on 8^{th} day and it reduced to 3 and that was maintained till 14^{th} day after immunization. In control (GB+IGN) cockerels a peak log_2 titre of 6 was reached on day 10 and that titre remained unchanged for 3 days and then declined. While, heat + (GB+IGN) cockerels showed peak log_2 titre of 6 on 8^{th} day which then immediately declined to 4 and was maintained till the end of the experiment.

Fig.4. Effect of heat stress on IgG titre

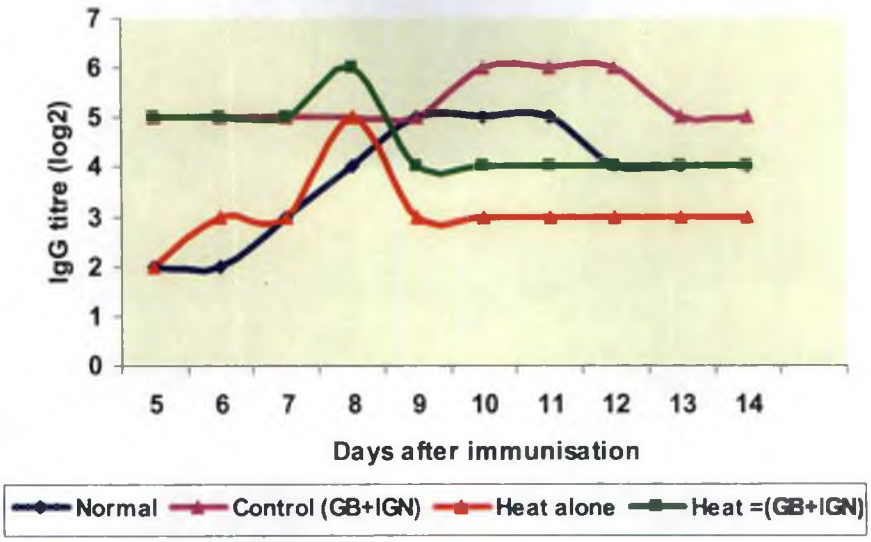
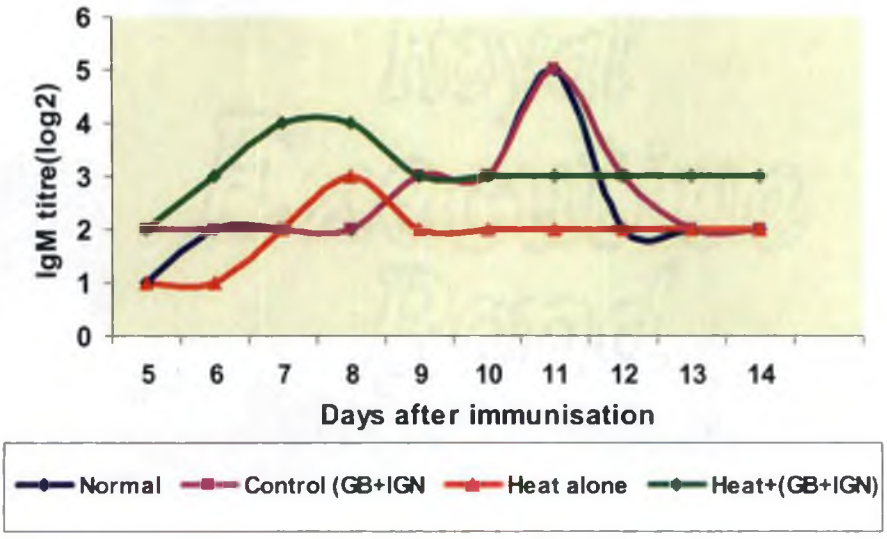


Fig.5.Effect of heat stress on IgM titre



4.5.4. Serum mercaptoethanol sensitive antibody (Ig M) level

The effect of heat stress on anti-sheep red blood cell (anti-SRBC) serum IgM titre in normal and GB+IGN treated cockerels was shown in Fig.5. The 5th day after immunization corresponds to first day of heat exposure. The $\log_2$ IgM titres of Group I (untreated-NHST i.e., normal) and Group III and V (untreated-5 and 10 days HST i.e., heat alone) cockerels on 5th day after immunization was 1, while a $\log_2$ titre value of 2 was exhibited by Group II (GB+IGN treated-NHST i.e., control (GB+IGN)) and Group V and IV (GB+IGN treated-5 and 10 days HST i.e., heat + (GB+IGN)) cockerels. Normal (untreated-NHST) cockerels showed a $\log_2$ titre value of 2 on 6th day, which gradually reached the peak $\log_2$ titre value of 5 on 11th day. It immediately declined to a $\log_2$ titre value of 2 and was maintained till the end of the experiment. In untreated-HST (heat alone) cockerels the highest $\log_2$ titre of 3 was reached on 8th day, which reduced immediately to 2, and that titre remained unchanged till 14th day after immunization. The control (GB+IGN) cockerels showed a peak $\log_2$ titre of 5 on day 10 which immediately declined to $\log_2$ titre value of 2 and was maintained till the end of the experiment. While in heat + (GB + IGN) cockerels, the highest $\log_2$ titre of 4 was reached on 7th day and remained unchanged for the next day, which then reduced to 3, and that titre remained unchanged through out the experiment.

4.6. EFFECT OF HEAT STRESS ON RELATIVE WEIGHT OF ADRENAL AND SPLEEN

4.6.1. The relative adrenal weight

The mean value of relative adrenal weight of Group I (untreated- NHST control) cockerels was 0.046 ± 0.001 g/100g bw, which did not show significant ($P>0.05$) difference with that of Group III (untreated-5 days HST) cockerels, while the mean value of Group V (untreated-10 days HST) cockerels showed a significant ($P<0.05$) difference with Group I. The mean value of adrenal weight in Group II (GB+IGN treated-NHST) cockerels was 0.047 ± 0.001 g/100g bw,

Table.5.Effect of heat stress on adrenal and spleen weight (Mean ± SE, n=8)

Groups	Relative adrenal weight (g/100g bw)	Relative spleen weight (g /100g bw)
I	0.046^{ab} ±0.001	0.111^{b}±0.001
II	0.047^{a} ±0.001	0.384^{a}±0.042
III	0.037^{bc} ±0.001	0.130^{b}±0.001
IV	0.044^{abc} ±0.001	0.319^{a}±0.007
V	0.034^{c} ±0.001	0.131^{b}±0.001
VI	0.043^{abc} ±0.001	0.151^{b}±0.001

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg bw for 10 days orally) and non- heat stressed controls.

Group III – Untreated and 5 days heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and 5 days heat stressed.

Group V – Untreated and 10 days heat stressed.

Group VI – (GB+IGN) treated and 10 days heat stressed.

which did not show any significant difference with GB+IGN treated groups of either 5 (Group IV) or 10 days (Group VI) HST cockerels (Table 5).

4.6.1. The relative spleen weight

There was no significant ($P>0.05$) change in the mean value of relative spleen weight of Group I (untreated-NHST) cockerels which showed value of 0.111± 0.001 g/100g bw when compared with Group III and V cockerels which were untreated 5 and 10 days HST cockerels exhibiting values of 0.130 ± 0.001 and 0.131 ± 0.001 g/100g bw respectively. The mean relative spleen weight of Group II (GB+IGN treated-NHST) cockerels was 0.384 ± 0.042 g/100g bw and this did not differ significantly with Group IV (GB+IGN treated-5 days HST) cockerels (0.319 ± 0.007 g/100g bw) but differed significantly ($P<0.05$) with that of Group VI (0.151 ± 0.001 g/100g bw) cockerels (Table 5).

4.7. EFFECT OF HEAT STRESS ON ADRENAL HISTOLOGY

In Group I (untreated-NHST) cockerels the adrenal histology showed the intermixing of adrenal cortex and medulla with no distinct regional distribution (Plate 4. Fig A). The Group II (GB+IGN treated-NHST) cockerels showed the zona fasciculata and medulla densely packed with cells (Plate 4. Fig. B). The Group III and V (untreated-5 and 10 days HST) cockerels showed increased fibrosis and depletion of lipid droplets in zona fasciculata (Plate 5. Fig.A), where as the Group V and VI (GB+IGN treated-5 and 10 days HST) cockerels exhibited moderate fibrosis and moderate number of lipid droplets in zona fasciculata (Plate 5. Fig.B).

4.8. EFFECT OF HEAT STRESS ON ANTIBODY FORMING CELLS

4.8.1. Jerne's plaque assay

On day 7 after immunization with rat red blood cells (RRBC) Group I (untreated-NHST i.e., normal) cockerels exhibited a maximum of 4.99×10^6 RFC/7.5×10^6 spleenocytes, which corresponds to 66.53 per cent

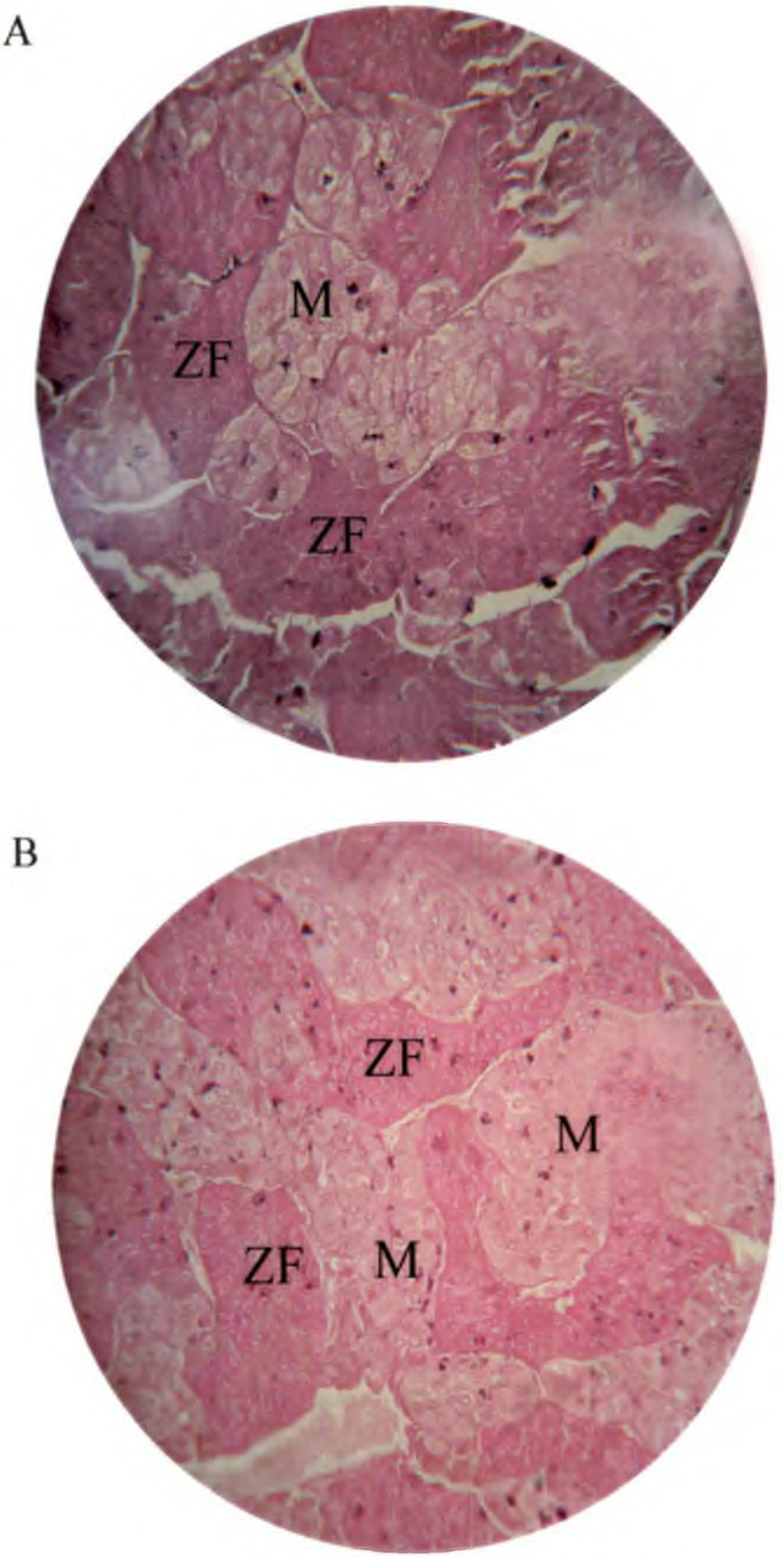


Plate 4. Adrenal histology of non heat stressed cockerels (400X)

A. Adrenals of untreated, non heat stressed cockerels.

B. Adrenals of (GB+IGN) treated, non heat stressed cockerels.

ZF- Zona Fasciculata M- Medulla

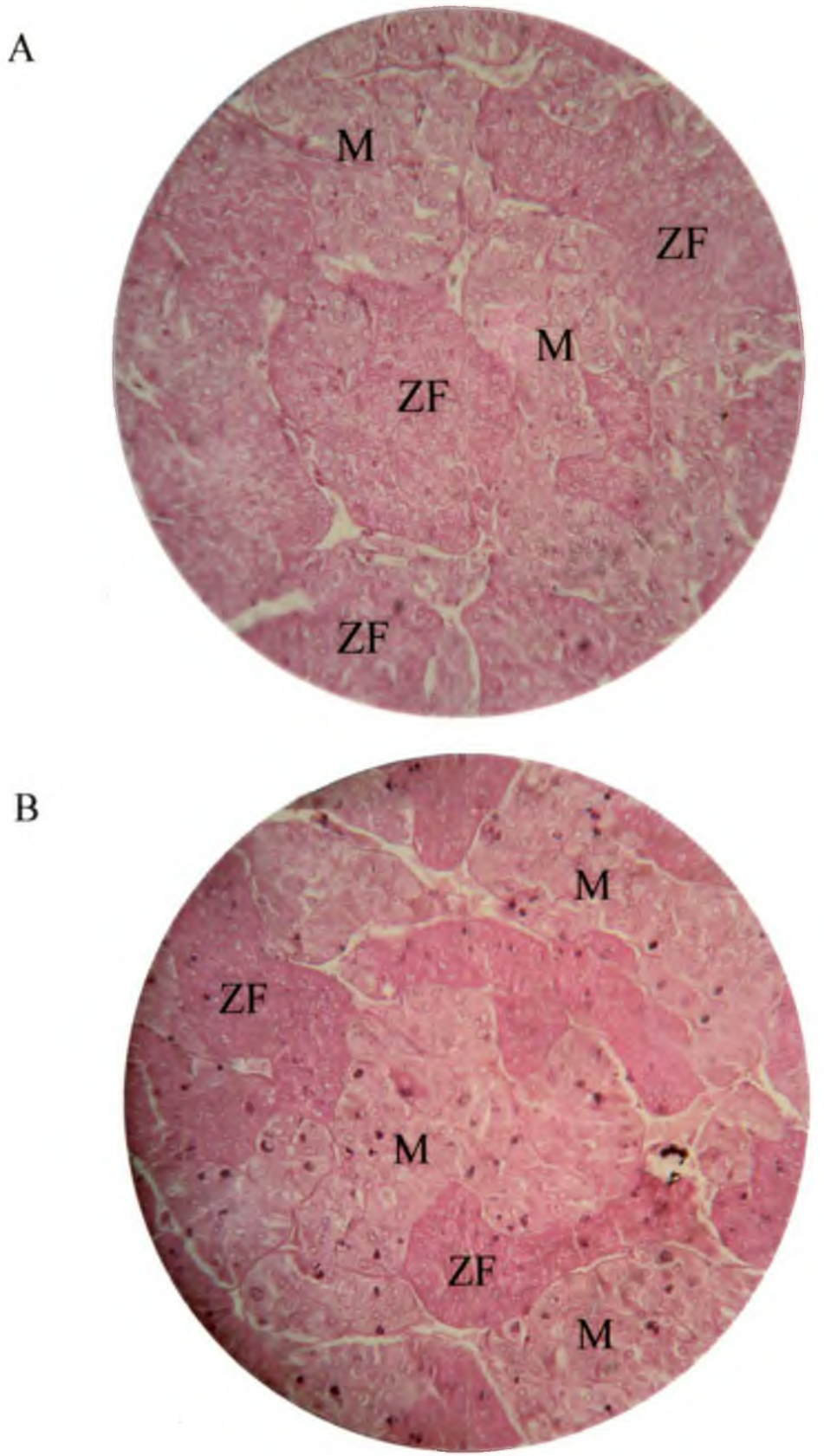


Plate 5. Effect of heat stress on adrenal histology (400X)

A. Adrenals of untreated, heat stressed cockerels

B. Adrenals of (GB+IGN) treated, heat stressed cockerels

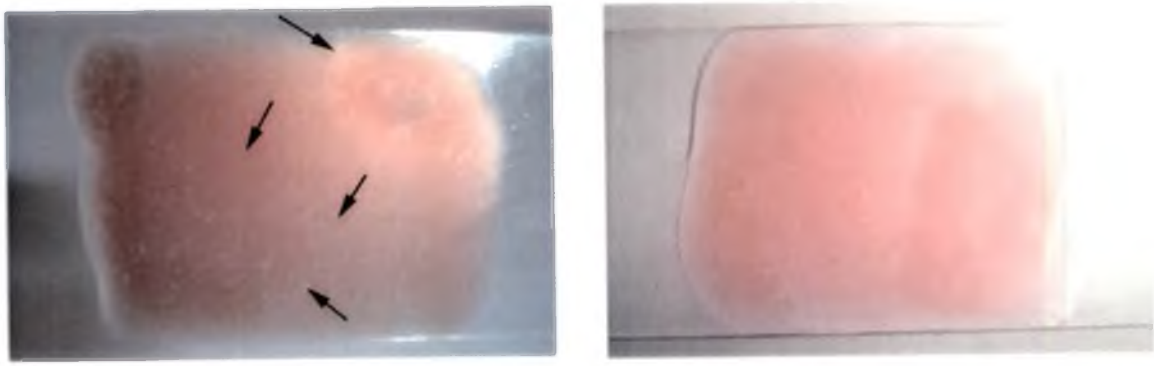
ZF- Zona Fasciculata M- Medulla

spleenocytes exhibiting plaque forming capability (Fig. 6. and Table.6). Similar response was exhibited by Group II (GB+IGN treated-NHST i.e., control), Group III (untreated-HST i.e., heat alone) and Group IV (GB+IGN treated-HST i.e., heat + (GB+IGN) cockerels which all showed a peak plaque forming response on 7^{th} day. Compared to all other groups the Group II cockerels responded maximally by 84.00% ($6.3x10^6/7.5x10^6$) spleenocytes exhibiting plaque-forming capability on 7^{th} day (Plate 6). However, from day 7 onwards cockerels showed comparatively lower PFC response.

4.8.2. Rosette- forming cell (RFC) immunocytoadherence assay

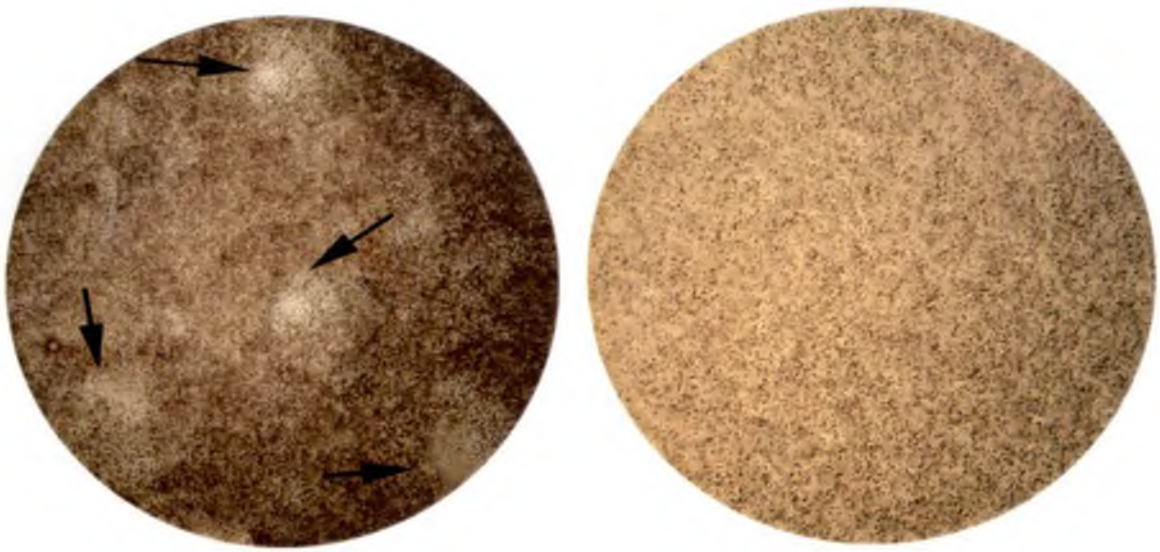
On day 7 after immunization with rat red blood cells (RRBC) Group I (untreated-NHST i.e., normal) cockerels exhibited a maximum of $4.8x10^6$ rosette forming cells (RFC)/ $7.5x10^6$ spleenocytes, which corresponds to 64 per cent spleenocytes exhibiting rosette forming capability (Fig. 7. and Table.7). Similar response was exhibited by Group II (GB+IGN treated-NHST i.e., control), Group III (untreated-HST i.e., heat alone) and Group IV (GB+IGN treated-HST i.e., heat + (GB+IGN) cockerels which all showed a peak rosette forming response on day 7 (Plate 6.Fig.A). Compared to all other groups the Group II cockerels responded maximally by 89.20% ($6.69x10^6$ out of $7.5x10^6$) spleenocytes exhibiting rosette forming capability on 7^{th} day. While Group III cockerels showed comparatively lower RFC response through out the experiment (Plate 6. Fig.B).

A



(i) (ii)

B



(iii) (iv)

Plate.6. Jerne's Plaque Assay

A. Macroscopic view of plaques in agar gel

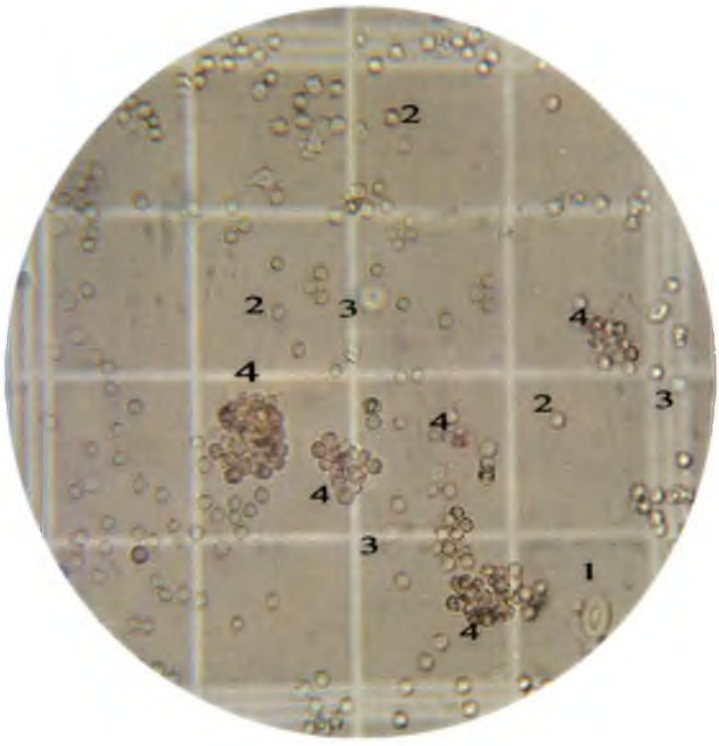
B. Microscopic view of plaques in agar gel

(i) & (iii) on 7th day after immunization

(ii) & (iv) on 11th day after immunization

Arrows indicate the site of hemolytic areas (plaques)

A



B

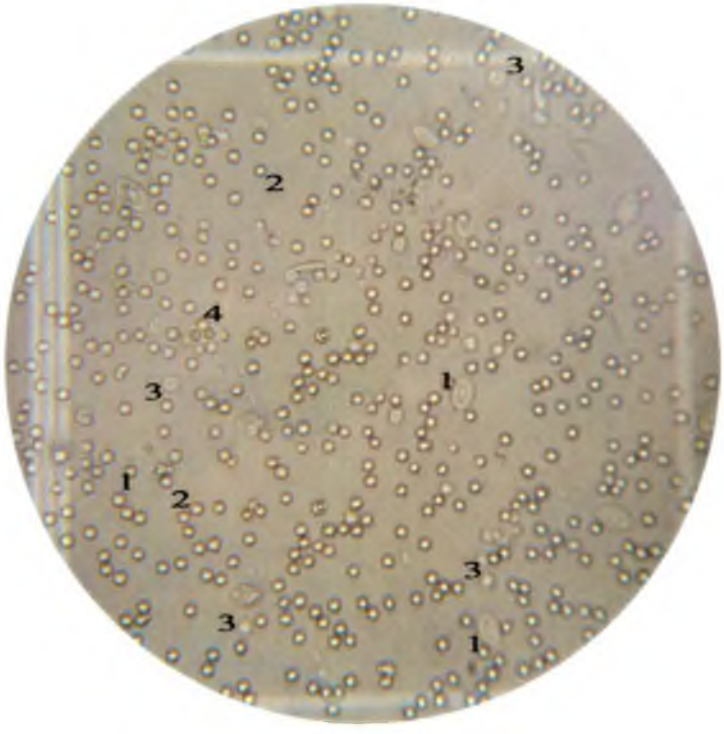


Plate.7. Rosette-forming cell (RFC) immunocytoadherence assay

Microscopic view of rosette forming cells

A. On 7th day after immunization

B. On 11th day after immunization

1. Chicken red blood cells
2. Rat red blood cells
3. Spleenocytes
4. Rosette forming cells

Fig.6.Effect of heat stress on spleenic plaque forming cells (PFC)

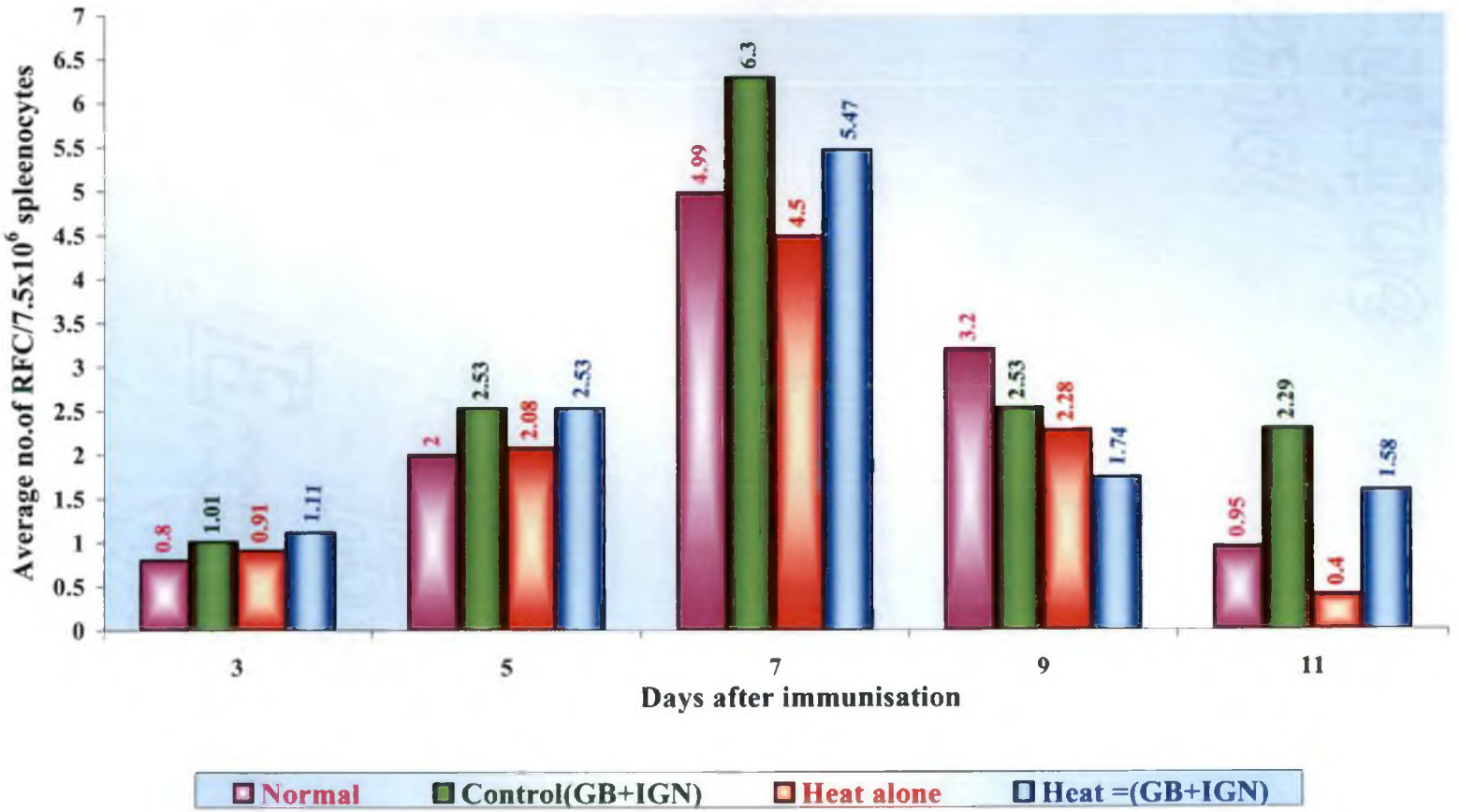


Fig.7.Effect of heat stress on rosette forming cells (RFC)

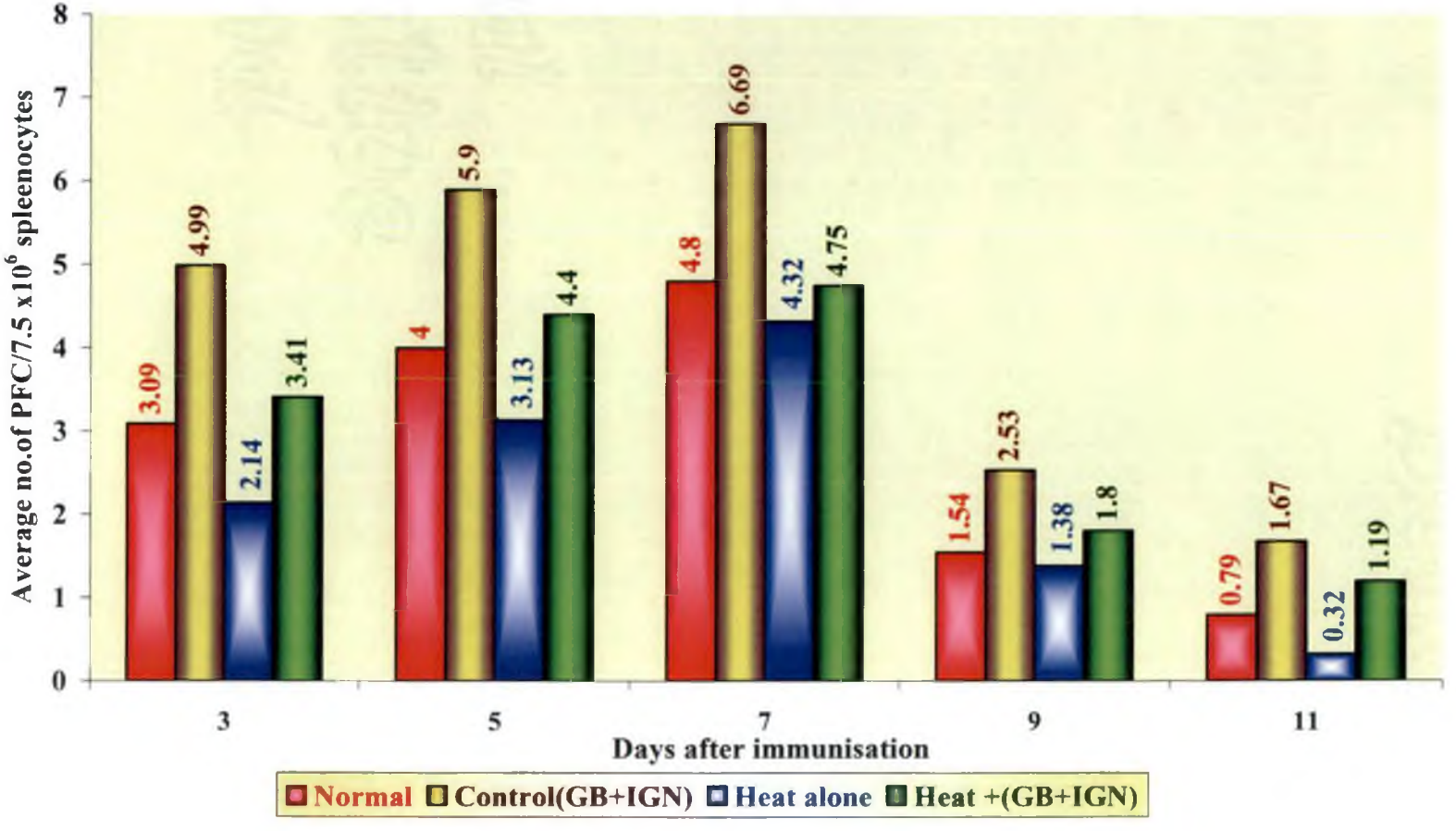


Table 6. Effect of heat stress on plaque forming cells (PFC) (n=5)

Groups	Days after immunization with Rat red blood cells (RRBC)				
	3	5	7	9	11
I	10.67%	26.67%	66.53%	42.67%	12.67%
II	13.47%	33.73%	84.00%	33.73%	30.53%
III	12.13%	27.73%	60.00%	30.40%	5.33%
IV	14.80%	33.73%	72.93%	23.20%	21.06%

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg.bw for 20 days orally) and non- heat stressed controls.

Group III – Untreated and heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and heat stressed.

Table 7. Effect of heat stress on rosette forming cells (RFC) (n=5)

Groups	Days after immunization with Rat red blood cells (RRBC)				
	3	5	7	9	11
I	41.20%	53.30%	64.00%	20.50%	10.53%
II	66.53%	78.66%	89.20%	33.73%	22.27%
III	28.53%	41.73%	57.6%	18.40%	4.27%
IV	45.47%	58.67%	63.33%	24.00%	15.86%

Group I – Untreated and non-heat stressed controls.

Group II – (GB+IGN) treated (2 g/kg.bw for 20 days orally) and non- heat stressed controls.

Group III – Untreated and heat stressed. (40±1°C, RH 60-70% for 4 h/day)

Group IV – (GB+IGN) treated and heat stressed.

4.9. EFFECT OF COLD STRESS ON BODY TEMPERATURE

Body temperature was found to be significantly ($P<0.05$) decreased in Group VII (untreated-5 days CST), IV (GB+IGN treated-5 days CST), V (untreated-10 days CST) and VI (GB+IGN treated-5 days CST) cockerels when compared to Group I and II untreated-NCST and GB+IGN treated-NCST cockerels. In CST cockerels, the body temperature reached 42.00 ±0.20°C by the end of 4h exposure to cold, while control birds recorded rectal temperature of 42.90±0.20°C. The CST cockerels lost an average of 40-45 g body weight daily upon 4h exposure to 40.00±1°C, which accounts for an average loss of 4% of body weight where as Group II (GB+IGN treated-NCST) cockerels did not show any change in the body weight. Cockerels exhibited a great deal of 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.

4.10. EFFECT OF COLD STRESS ON CERTAIN HAEMATOLOGICAL PARAMETERS

4.10.1. Haemoglobin concentration (Hb)

The mean Hb concentration for Group I (untreated-NCST) control cockerels was 10.63 ± 0.23 g per cent, and it increased significantly ($P<0.05$) to 11.19 ± 0.23 g per cent in Group VII (untreated-5 days CST) and then decreased significantly ($P<0.05$) to 10.00 ± 0.23 g per cent, in Group IX (untreated-10 days CST) cockerels. The mean Hb concentration of Group II (GB+IGN treated-NCST) cockerels was 10.06 ± 0.02 g per cent and it increased significantly ($P<0.05$) to 11.56 ± 0.36 g per cent in Group VIII (GB+IGN treated-5 days CST) cockerels., where as Group II cockerels did not show any significant difference with Group X (GB+IGN treated-10 days CST) cockerels with mean Hb concentration of 10.88 ± 0.29 g per cent (Table 8).

Table 8. Effect of cold stress on certain haematological parameters (Mean ± SE, n=8)

Groups	Haemoglobin (Hb) g%	Packed cell volume (PCV) %	Total leucocyte count (TLC) x $10^3/\mu l$	Lymphocytes (L) %	Heterophils (H) %	H/L Ratio
I	$10.63^{b} \pm 0.23$	$34.30^{bc} \pm 1.34$	$10975.00^{ab} \pm 741$	$59.63^{a} \pm 4.65$	$40.38^{c} \pm 4.65$	$0.77^{c} \pm 0.13$
II	$10.06^{bc} \pm 0.18$	$32.15^{c} \pm 0.99$	$12550.00^{a} \pm 72$	$60.75^{a} \pm 0.75$	$39.13^{c} \pm 0.87$	$0.63^{c} \pm 0.28$
VII	$11.19^{a} \pm 0.23$	$41.76^{a} \pm 2.07$	$8562.00^{d} \pm 566$	$35.38^{b} \pm 2.23$	$64.63^{b} \pm 1.29$	$2.36^{a} \pm 0.25$
VIII	$11.56^{a} \pm 0.36$	$41.56^{a} \pm 0.51$	$8843.00^{cd} \pm 513$	$30.88^{b} \pm 2.23$	$69.13^{a} \pm 2.21$	$1.86^{b} \pm 0.11$
IX	$10.00^{c} \pm 0.23$	$37.59^{b} \pm 0.72$	$10012.00^{b} \pm 456$	$33.25^{b} \pm 1.41$	$69.25^{a} \pm 2.33$	$2.38^{a} \pm 0.24$
X	$10.88^{ab} \pm 0.29$	$35.43^{bc} \pm 0.59$	$11196.00^{ab} \pm 741$	$30.75^{b} \pm 2.33$	$66.75^{b} \pm 1.41$	$2.00^{b} \pm 0.09$

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non- cold stressed control.
Group II – (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.
Group VII – Untreated and 5 days cold stressed. (8±1°C, RH 40-50% for 4 h/day)
Group VIII – (GB+IGN) treated and 5 days cold stressed.
Group IX – Untreated and 10 days cold stressed.
Group X – (GB+IGN) treated and 10 days cold stressed.

4.10.2. Packed cell volume (PCV)

The mean value of PCV in Group VII (untreated-5 days CST) cockerels was 41.76 ± 2.07 per cent and it was significantly (P<0.05) higher than that in Group I (untreated-NCST) cockerels (34.30± 1.34 per cent), later on decreased significantly (P<0.05) to reach 37.59± 0.72 per cent in Group IX (untreated-10 days CST) cockerels. Group II (GB+IGN treated-NCST) cockerels showed a mean PCV value of 32.15 ± 0.99 per cent, which increased significantly to 41.56 ± 0.51 per cent in Group VIII (GB+IGN treated-5 days CST) where as the PCV value decreased significantly (P<0.05) to 35.43 ± 0.59 per cent in Group X (GB+IGN treated-10 days CST) cockerels (Table 8).

4.10.3. Total leucocyte count (WBC count)

The mean total leucocyte count of Group I (untreated-NCST) cockerels was 10975.00 ± 741 /µl and it decreased significantly (P<0.05) to 8562.00 ± 564/µl in Group VII (untreated-5 days CST) cockerels, whereas no significant difference was noticed with that in Group IX (untreated-10 days CST) cockerels when compared with Group I cockerels. The Group II (GB+IGN treated-NCST) cockerels exhibited a mean leucocyte count of 12550.00 ± 72/µl and it decreased significantly (P<0.05) to 8843.00 ± 513/µl in Group VIII (GB+IGN untreated-5 days CST) cockerels whereas no significant difference was noticed with that in Group X (GB+IGN treated-10 days CST) cockerels when compared with Group II cockerels (Table 8).

4.10.4. Lymphocyte (L) count

Group I (untreated-NCST) cockerels possessed a mean lymphocyte count of 59.63 ± 4.65 per cent. This value was significantly (P<0.05) higher than those exhibited by Group VII (untreated-5 days CST) and Group IX (untreated-10 days CST) and it showed 35.38± 2.23 and 33.25 ± 1.41 per cent respectively. The mean lymphocyte count of Group II (GB+IGN treated-NCST) cockerels was 60.75± 0.75 per cent and it decreased significantly (P<0.05) to 30.88 ± 2.23 per

cent in Group VIII (GB+IGN treated-5 days CST) and to 30.75 ± 2.33 per cent in Group X (GB+IGN treated-10 days CST) cockerels (Table 8).

4.10.5. Heterophil (H) count

The mean heterophil count for Group I (untreated-NCST) cockerels was 40.38± 4.65 per cent, and it increased significantly ($P<0.05$) to a higher value of 64.63 ± 1.29 per cent and 69.25 ± 2.33 per cent in Group VII and Group IX cockerels respectively. Similarly, there was a significant ($P<0.05$) increase in mean heterophil count of Group VIII (69.13± 2.21 per cent) and Group X (66.75 ± 1.41 per cent) when compared to Group II (39.13± 0.87 per cent) cockerels (Table 8).

4.10.6. Heterophil/ Lymphocyte (H/L) ratio

The mean H/L ratio in Group I (untreated-NCST) cockerels was 0.77 ± 0.13, and it increased significantly ($P<0.05$) to 2.36 ± 0.25 in Group VII, (untreated-5 days CST) and 2.38 ± 0.25 in Group IX (untreated-10 days CST) cockerels. The mean H/L ratio in Group II (GB+IGN treated-NCST) cockerels was 0.63 ± 0.28, and it increased significantly ($P<0.05$) to 1.86 ± 0.11 and 2.00 ± 0.09 in Group VIII and Group X cockerels respectively (Table 8).

4.11. EFFECT OF COLD STRESS ON SERUM PROTEIN PROFILE

4.11.1. Serum protein profile:

4.11.1.1. *Serum total protein content*

The mean serum total protein content in Group I (untreated-NCST) cockerels was 5.38 ± 0.53 g/dl and it did not show any significant ($P>0.05$) difference with untreated groups of either 5 or 10 days CST cockerels. The mean serum total protein content in Group II (GB+ IGN treated-NCST) cockerels was 5.50 ± 0.27 g/dl and it also did not show any significant difference with Group VIII (GB+IGN treated-5 days CST) cockerels, whereas it increased significantly

Table 9.Effect of cold stress on serum protein profile (Mean ± SE, n=8)

Groups	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
I	5.38^{b}± 0.53	2.71^{c}±0.22	2.09^{a} ± 0.13
II	5.50^{b} ± 0.27	3.40^{c}±0.17	2.54^{a} ±0.43
VII	6.00^{b} ± 0.19	4.01^{b}±0.35	1.69^{a} ± 0.14
VIII	6.25^{ab} ± 0.25	4.77^{b}±0.23	1.86^{a} ± 0.22
IX	5.88^{b} ± 0.23	4.73^{b}±0.13	1.53^{a} ± 0.28
X	7.13^{a}± 0.44	5.13^{a}±0.33	1.50^{a} ± 0.18

Mean ± SE having different superscripts, differ significantly (P<0.05) between groups

Group I – Untreated and non- cold stressed control.

Group II – (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.

Group VII – Untreated and 5 days cold stressed. (8±1°C, RH 40-50% for 4 h/day)

Group VIII – (GB+IGN) treated and 5 days cold stressed.

Group IX – Untreated and 10 days cold stressed.

Group X – (GB+IGN) treated and 10 days cold stressed.

($P<0.05$) to 7.13 ± 0.44 g/dl in Group X (GB+IGN treated-10 days CST) cockerels (Table 9).

4.11.1.2. *Albumin content*

The mean serum albumin content in Group I (untreated-NCST) cockerels was 2.71 ± 0.22 g/dl, and it increased significantly ($P<0.05$) to 4.01 ± 0.35 g/dl in Group VII (untreated-5 days CST) and 4.73 ± 0.13 g/dl in Group IX (untreated-10 days CST) cockerels. The mean serum albumin content in Group II (GB+IGN treated-NCST) cockerels was 3.40 ± 0.17 g/dl, and it increased significantly ($P<0.05$) in Group VIII (GB+IGN treated-5 days CST) cockerels (4.77 ± 0.23g/dl) and further increased significantly ($P<0.05$) to 5.13 ± 0.33 g/dl in Group X (GB+IGN treated-10 days CST) cockerels (Table 9).

4.11.1.3. *Globulin content*

The mean serum globulin content of all the birds in different groups did not differ significantly ($P>0.05$) either by cold stress and GB+IGN treatment or by both. The values ranged from 1.50 ± 0.18 g/dl in Group X (GB+IGN treated-10 days CST) cockerels to 2.54±0.43 g/dl in Group II (GB+IGN treated-NCST) cockerels (Table 9).

4.11.2. Serum enzyme activities

4.11.2.1. *Serum lactate dehydrogenase (LDH) activity*

The mean LDH activity in Group I (untreated-NCST) cockerels was 1374.75 ± 4.02 U/l. A significant ($P<0.05$) decrease to 350.13± 4.65 U/l in Group VII (untreated-5 days CST) cockerels and to 759.50 ± 4.63 U/l in Group IX (untreated-10 days CST) cockerels was observed. In Group II (GB+IGN treated-NCST) cockerels the LDH activity was 1082.13 ± 11.47 U/l, and it decreased significantly to 501.63± 1.99 U/l in Group VIII and to 567.75 ± 4.24 U/l in Group X (GB+IGN treated-10 days CST) cockerels (Table 10).

4.11.2.2. *Serum Creatine kinase (CK) activity*

The mean serum CK activity in Group I (untreated-NCST) cockerels was 1268.25 ± 6.47 U/l and it increased significantly (P<0.05) to 1859 ± 16.27 U/l in Group VII (untreated-5 days CST) cockerels. Further significant (P<0.05) elevation to 2152.13 ± 19.76 U/l was observed in Group IX (untreated-10 days CST) cockerels. The mean serum CK activity in Group II (GB+IGN treated- NCST) cockerels was 1583.88 ± 5.49 U/l and it decreased significantly (P<0.05) to 1198.00 ± 12.53 U/l in Group VIII (GB+IGN treated-5 days CST) cockerels. Later it increased significantly (P<0.05) to 2062.25 ± 51.83 U/l in Group X (GB+IGN treated 10 days) CST cockerels (Table 10).

4.11.3. Serum electrolytes level

4.11.3.1. *Serum sodium level*

There was no significant (P>0.05) difference in the mean serum sodium content of cockerels in different groups. The results showed that cold stress and GB+IGN treatment or both could not bring about any significant variation in the serum sodium level. The values ranged from 133.55 ± 5.03 in Group I (untreated- NCST) cockerels to150.28 ± 4.51 in Group IX (untreated-10 days CST) cockerels (Table 10).

4.11.3.2. *Serum potassium level*

The mean serum potassium level in Group I (untreated-NCST) cockerels was 4.64 ± 0.12 mEq/l and it significantly (P<0.05) increased to 5.50± 0.06 mEq/l in Group VII (untreated-5 days CST) cockerels. Later on, it decreased significantly to 5.15± 0.01 mEq/l in Group IX (untreated-10 days CST) cockerels. The mean serum potassium level in Group II (GB+IGN treated-NCST)) cockerels was 5.03 ± 0.18 mEq/l and it significantly (P<0.05) increased to 5.50 ± 0.03 mEq/l and 5.64 ± 0.06 mEq/l in Group VIII (GB+IGN treated-5 days CST) and Group X (GB+IGN treated-10 days CST) cockerels respectively (Table 10).

Table 10. Effect of cold stress on certain serum enzyme activities and electrolyte levels (Mean ± SE, n=8)

Groups	Lactate dehydrogenase (LDH)(U/l)	Creatine kinase (CK)(U/l)	Serum Sodium (mEq/L)	Serum Potassium (mEq/L)
I	$1374.75^{a} \pm 4.02$	$1268.25^{e} \pm 6.47$	$133.55^{a} \pm 5.03$	$4.64^{c} \pm 0.12$
II	$1082.13^{b} \pm 11.47$	$1583.88^{d} \pm 5.49$	$146.34^{a} \pm 7.39$	$5.03^{b} \pm 0.18$
VII	$350.13^{f} \pm 4.65$	$1859.38^{c} \pm 16.27$	$147.85^{a} \pm 1.69$	$5.50^{a} \pm 0.06$
VIII	$501.63^{e} \pm 1.99$	$1198.00^{f} \pm 12.53$	$145.96^{a} \pm 3.38$	$5.50^{a} \pm 0.03$
IX	$759.50^{c} \pm 4.63$	$2152.13^{a} \pm 19.76$	$150.28^{a} \pm 4.51$	$5.15^{b} \pm 0.01$
X	$567.75^{d} \pm 4.24$	$2062.25^{b} \pm 51.83$	$149.79^{a} \pm 5.50$	$5.64^{a} \pm 0.06$

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non- cold stressed control.

Group II – (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.

Group VII – Untreated and 5 days cold stressed. (8±1°C, RH 40-50% for 4 h/day)

Group VIII – (GB+IGN) treated and 5 days cold stressed.

Group IX – Untreated and 10 days cold stressed.

Group X – (GB+IGN) treated and 10 days cold stressed.

4.11.4. Serum C-reactive protein (CRP) level

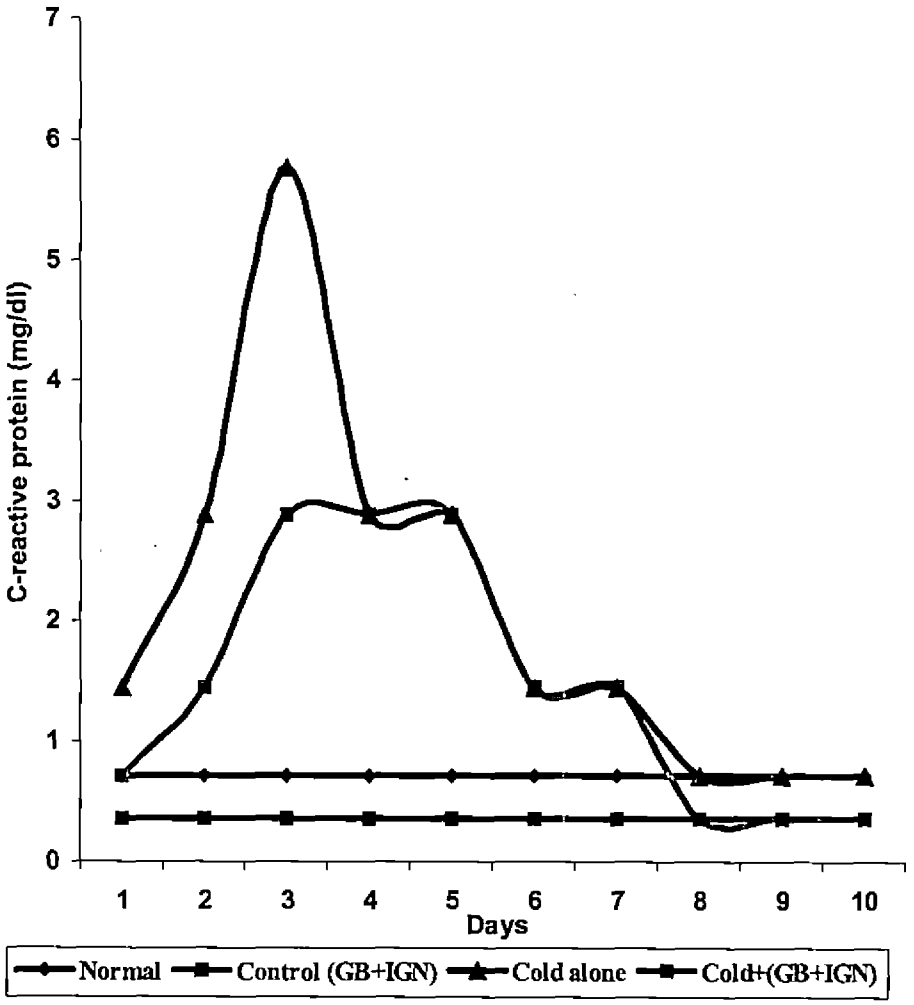
The effect of cold stress on serum CRP level in normal and GB+IGN treated cockerels was shown in Fig.8. Both the Group I (untreated-NCST i.e., normal) and Group II (GB+IGN treated-NCST i.e., control (GB+IGN)) cockerels maintained a stable value of 0.72 and 0.36 mg/dl respectively throughout the experiment. However, on the first day of cold exposure Group VII and IX (untreated-5 and 10 days CST i.e., cold alone) cockerels exhibited a value of 1.44 mg/dl, and it was two times higher than the Group I and four times higher than the Group II cockerels, where as Group VIII and X (GB+IGN treated-5 and 10 days CST i.e., cold+ (GB+IGN)) cockerels exhibited a value of 0.72 mg/dl. In Group VII and IX (untreated-5 and 10 days CST i.e., cold alone) cockerels, a peak value of 5.76 mg/dl was observed on 3rd day of cold exposure and thereafter the value declined immediately to 2.88 mg/dl and then slowly returned to a value of 0.72 mg/dl at the end of the experiment. Cold + (GB+IGN) cockerels exhibited a value of 1.44 mg/dl on 2nd day of cold exposure, and then increased to attain a peak level of 2.88 mg/dl on 3rd day of cold exposure. The peak was maintained for 3 days and then decreased to 1.44 mg/dl, and finally reached 0.36 mg/dl and remained unchanged till the end of the experiment.

4.12. EFFECT OF HEAT STRESS ON CERTAIN SERUM HORMONES LEVEL

4.12.1. Serum cortisol concentration

The mean serum cortisol concentration in Group I (untreated-NCST) cockerels was 32.00 ± 0.71 ng/ml and it decreased significantly ($P<0.05$) to 28.63± 0.81 ng/ml and 26.56± 0.28 ng/ml in Group VII and IX (untreated-5 and 10 days CST) cockerels respectively. However, the mean serum cortisol concentration in Group II (GB+IGN treated-NCST) cockerels was 27.50 ± 0.52 ng/ml, and it did not differ significantly in Group IX (GB+IGN treated-5 days CST) cockerels with mean serum cortisol concentration of 25.00 ± 0.60 ng/ml.

Fig.8.Effect of cold stress on serum C-reactive protein (CRP) level



Further the level decreased significantly (P<0.05) to 19.50 ± 0.91 ng/ml in Group X (GB+IGN treated-10 days CST) cockerels (Table 11).

4.12.2. Serum T_3 concentration

The mean serum T_3 concentration in Group I (untreated-NCST) cockerels was 106.00± 1.93 ng/dl and it decreased non significantly (P>0.05) to 63.50 ± 3.92 ng/dl in Group VII (untreated-5 days CST) cockerels. Further significant (P<0.05) elevation to 220.13 ± 24.61 ng/dl was observed in Group IX (untreated-10 days CST) cockerels. The mean serum T_3 level in Group II (GB+IGN treated- NCST) cockerels was 44.25 ± 1.03ng/dl and it increased significantly (P<0.05) to 176.13 ± 23.08 ng/dl in Group VIII (GB+IGN treated-5 days CST) cockerels. Later it increased non significantly (P<0.05) to 220.63 ± 18.11 ng/dl in Group X (GB+IGN treated-10 days) CST cockerels (Table 11).

4.12.3. Serum T_4 concentration

The mean serum T_4 concentration decreased significantly (P<0.05) to 2.63 ± 1.10 µg/dl in Group VII (untreated-5 days CST) cockerels when compared to 3.55 ± 0.24 µg/dl exhibited by Group I (untreated-NCST) cockerels. While the T_4 level in Group IX (untreated-10 days CST) cockerels was 2.41± 0.63 µg/dl, and it showed no significant (P>0.05) difference with that in Group I. The mean serum T_4 concentration in Group II (GB+IGN treated-NCST) cockerels was 3.49 ± 0.29 µg/dl, which decreased significantly (P<0.05) to 2.63 ± 0.57 µg/dl in Group VIII (GB+IGN treated-5 days CST) and it later increased significantly to 3.31 ± 0.13 µg/dl in Group X (GB+IGN treated-10 days CST) cockerels respectively (Table11).

Table .11.Effect of cold stress on serum hormone profile (Mean ± SE, n=8)

Groups	Serum Cortisol (ng/ml)	Serum T_3 (ng/dl)	Serum T_4 (µg/dl)
I	$32.00^{a} \pm 0.71$	$106.00^{b} \pm 1.93$	$3.55^{a} \pm 0.24$
II	$27.50^{b} \pm 0.52$	$44.25^{c} \pm 1.03$	$3.49^{a} \pm 0.29$
VII	$28.63^{b} \pm 0.81$	$63.50^{bc} \pm 3.92$	$2.63^{b} \pm 1.10$
VIII	$25.00^{b} \pm 0.60$	$176.13^{a} \pm 23.08$	$2.63^{b} \pm 0.57$
IX	$26.56^{b} \pm 0.28$	$220.13^{a} \pm 24.61$	$2.41^{b} \pm 0.63$
X	$19.50^{c} \pm 0.91$	$220.63^{a} \pm 18.11$	$3.31^{a} \pm 0.13$

Mean ± SE having different superscripts, differ significantly ($P<0.05$) between groups

Group I – Untreated and non- cold stressed control.

Group II – (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.

Group VII – Untreated and 5 days cold stressed. (8±1°C, RH 40-50% for 4 h/day)

Group VIII – (GB+IGN) treated and 5 days cold stressed.

Group IX – Untreated and 10 days cold stressed.

Group X – (GB+IGN) treated and 10 days cold stressed.

4.13. EFFECT OF COLD STRESS ON IMMUNE RESPONSES

4.13.1. Haemaglutinin (HA) assay

The effect of cold stress on serum anti-sheep red blood cell (anti-SRBC) haemagglutinin (HA) titre in normal and GB+IGN treated cockerels has been illustrated in Fig.9.The 5th day after immunization corresponds to first day of cold exposure. The $\log_2$ HA titre was 3 for Group I (untreated-NCST i.e., normal) and untreated-CST (cold alone) cockerels on 5th day after immunization, while a $\log_2$ titre value of 7 was exhibited by Group II (GB+IGN treated-NCST i.e., control (GB+IGN)) and Group VII and IX (GB+IGN treated-CST i.e., cold + (GB+IGN)) cockerels. On 11th day after immunization the peak $\log_2$ titre values of 10 and 11 was observed in normal and control (GB+IGN) cockerels respectively. Thereafter the $\log_2$ titre values decreased to 6 in normal and to 7 in control (GB+IGN) cockerels and it persisted till the end of the experiment. While in untreated-CST cockerels (cold alone) the peak $\log_2$ titre value of 11 was observed on day 11 that declined to $\log_2$ titre of 8 and finally reached a $\log_2$ titre of 6 by the end of experiment. However, in cold + (GB+IGN) cockerels a peak $\log_2$ titre value of 12 was encountered on day 11 which then immediately declined gradually to a $\log_2$ titre value of 8 and finally reached a $\log_2$ titre of 7 by the end of experiment.

4.13.2. Haemolysin (HL) response

The effect of cols stress on serum anti-sheep red blood cell (anti-SRBC) haemolysin (HL) titre of cockerels exposed to cold has been illustrated in Fig.10. The 5th day after immunization corresponds to first day of cold exposure. The $\log_2$ HL titre was 2 for Group I (untreated-NCST i.e., normal) and Group VII and IX (untreated-CST i.e., cold alone) cockerels on 5th day after immunization, while a $\log_2$ titre value of 6 was exhibited by Group II (GB+IGN treated-NCST i.e., control (GB+IGN)) and Group VIII and X (GB+IGN treated-CST i.e., cold + (GB+IGN)) cockerels. On 11th day after immunization the peak $\log_2$ titre values of 9 and 10 was observed in normal and control (GB+IGN) cockerels respectively. Thereafter the $\log_2$ titre values decreased to 5 in normal and to 6 in

Fig.9. Effect of cold stress on haemagglutinin (HA) titre

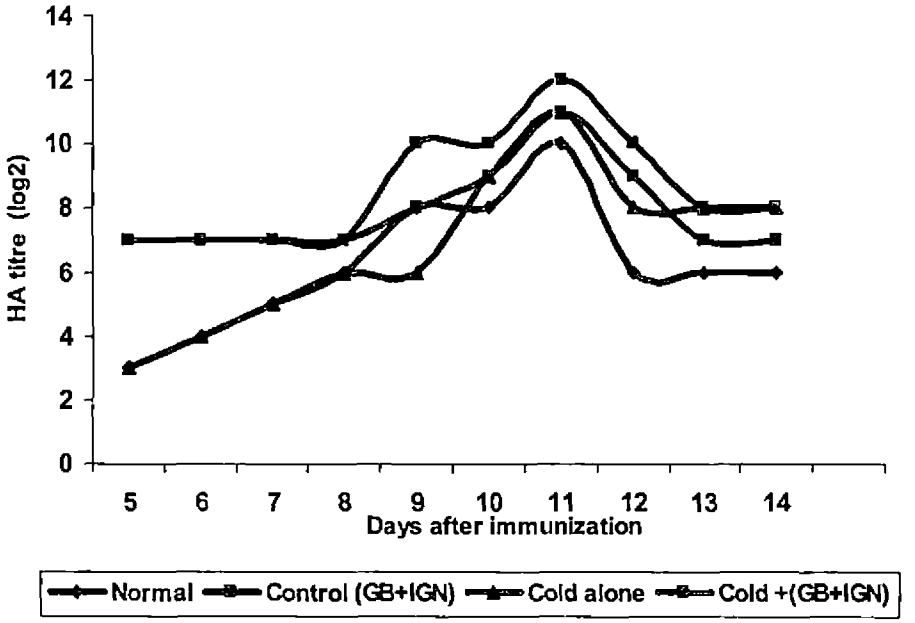
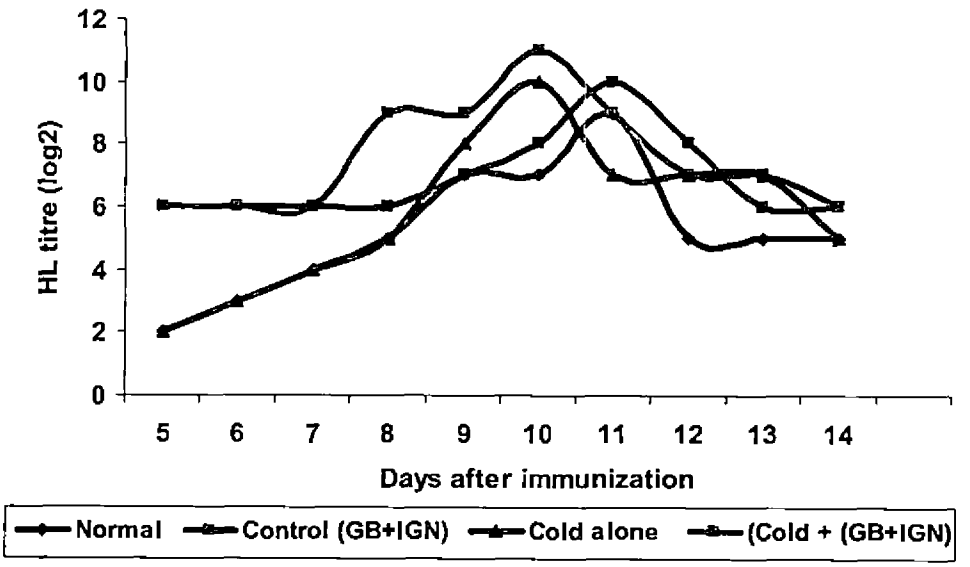


Fig.10. Effect of cold stress on Haemolysin (HL) titre



control (GB+IGN) cockerels and that persisted till the end of the experiment. While in untreated-CST cockerels (cold alone) the peak $\log_2$ titre value of 10 was observed on day 11 and it declined to $\log_2$ titre of 7 for next 3 days and the reached a $\log_2$ titre of 5 by 14th day after immunization. However, in cold + (GB+IGN) cockerels a peak $\log_2$ titre value of 11 was encountered on day 10 which then immediately declined to a $\log_2$ titre value of 9 and it declined to $\log_2$ titre of 7 for next 2 days and the reached a $\log_2$ titre of 6 by 14th day after immunization.

4.13.3. Serum mercaptoethanol resistant antibody (Ig G) level

The effect of cold stress on anti-sheep red blood cell (anti-SRBC) serum IgG titre in normal and GB+IGN treated cockerels was shown in Fig.11. The 5th day after immunization corresponds to first day of cold exposure. The $\log_2$ IgG titres of Group I (untreated-NCST i.e., normal) and Group VII and IX (untreated-CST i.e., cold alone) cockerels on 5th day after immunization was 2, while a $\log_2$ titre value of 5 was exhibited by Group II (GB+IGN treated-NCST i.e., control (GB+IGN)) and Group VIII and X (GB+IGN treated-CST i.e., cold + (GB+IGN)) cockerels. In normal cockerels, peak $\log_2$ titre value of 5 was observed on 9th day and that was maintained till day 11 and then it declined to a value of 4 by the end of the experiment. In untreated-CST (cold alone) cockerels the highest $\log_2$ titre of 5 was reached on 10th day and the peak titre remained for 5 days followed by a reduction to 4 on 14th day after immunization. In control (GB+IGN) cockerels a peak $\log_2$ titre of 6 was reached on day 9 and that titre remained unchanged for 3 more days. While cold + (GB+IGN) cockerels showed peak $\log_2$ titre of 7 on 11th day and it then immediately declined to 5 and was maintained till the end of the experiment.

4.13.4. Serum mercaptoethanol sensitive antibody (Ig M) level

The effect of cold stress on anti-sheep red blood cell (anti-SRBC) serum IgM titre in normal and GB+IGN treated cockerels was show Fig.12. The 5th day after immunization corresponds to first day of cold exposure. The $\log_2$ IgM titres

Fig 11.Effect of cold stress on IgG level

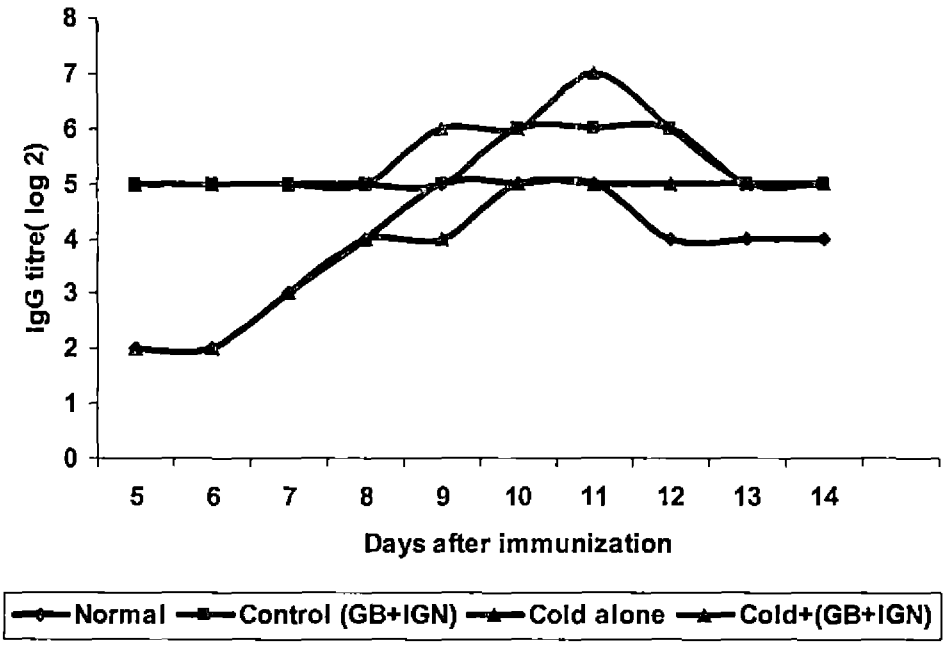
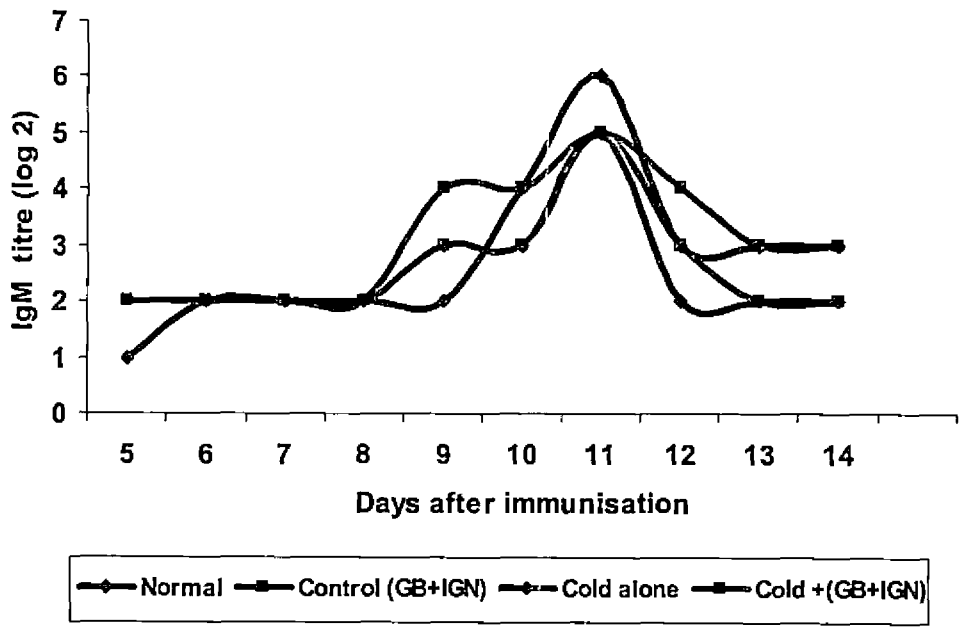


Fig. 12. Effect of cold stress on IgM level



of Group I (untreated-NCST i.e., normal) and Group VII and IX (untreated-CST i.e., cold alone) cockerels on 5th day after immunization was 1 while a log_2 titre value of 2 was exhibited by Group II (GB+IGN treated-NCST i.e., control (GB+IGN)) and Group VIII and X (GB+IGN treated-CST i.e., cold + (GB+IGN)) cockerels. Normal cockerels showed a log_2 titre value of 2 on 6th day, and it gradually reached the peak log_2 titre value of 5 on 11th day. It immediately declined to a log_2 titre value of 2 and was maintained till the end of the experiment. In untreated-CST (cold alone) cockerels the highest log_2 titre of 6 was reached on 11th day and it reduced gradually to log_2 titre value of 2 at the end of the experiment. Control (GB+IGN)] cockerels showed a peak log_2 titre of 5 on day 11 and it immediately declined to log_2 titre value of 2 and was maintained till the end of the experiment. While cold + (GB+IGN) cockerels showed a peak log_2 titre of 5 on day 11 and that titre immediately reduced to 2 at the end of the experiment.

4.14. EFFECT OF COLD STRESS ON RELATIVE WEIGHT OF ADRENAL AND SPLEEN

4.14.1. The relative adrenal weight

The mean value of relative adrenal weight of Group I (untreated NCST control) cockerels was 0.046 ± 0.001 g/100g bw, and it decreased non significantly ($P>0.05$) in Group VII (untreated-5 days CST) and Group IX (untreated-10 days CST) cockerels with 0.034 ± 0.001 and 0.030 ± 0.001 g/100g bw respectively. The mean value of adrenal weight in Group II (GB+IGN treated-NCST) cockerels was 0.047 ± 0.001 g/100g bw, and it did not show any significant difference with GB+IGN treated groups of either 5 or 10 days CST cockerels with 0.044 ± 0.001 and 0.047 ± 0.001 g/100g bw respectively (Table 12).

Table.12.Effect of cold stress on adrenal and spleen weight (Mean ± SE, n=8)

Groups	Relative adrenal weight (g /100g bw)	Relative spleen weight (g/100g bw)
I	0.046^{ab} ±0.001	0.111^{b}±0.001
II	0.047^{a} ±0.001	0.384^{a}±0.042
VII	0.034^{b} ±0.001	0.122^{d}±0.001
VIII	0.044^{a}±0.001	0.273^{b}±0.001
IX	0.030^{b} ±0.001	0.101^{f}±0.001
X	0.047^{a} ±0.001	0.139^{c}±0.014

Mean ± SE having different superscripts, differ significantly (P<0.05) between groups

Group I – Untreated and non- cold stressed control.

Group II – (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.

Group VII – Untreated and 5 days cold stressed. (8±1°C, RH 40-50% for 4 h/day)

Group VIII – (GB+IGN) treated and 5 days cold stressed.

Group IX – Untreated and 10 days cold stressed.

Group X – (GB+IGN) treated and 10 days cold stressed.

4.14.2. The relative spleen weight

The mean value of relative spleen weight of Group I (untreated-NCST control) cockerels was 0.111 ± 0.001 g/100g bw, and it differed significantly ($P<0.05$) with that of Group VII (untreated-5 days CST) and Group IX (untreated-10 days CST) cockerels with 0.122 ± 0.001 and 0.101± 0.001 g/100g bw respectively. Similarly, the mean value of relative spleen weight of Group II (GB+IGN treated-NCST) cockerels was 0.384 ± 0.042 g/100g bw, and it differed significantly ($P<0.05$) with that of Group VIII (GB+IGN treated-5 days CST) and Group X (GB+IGN treated-10 days CST) cockerels with 0.273± 0.001 and 0.139± 0.014 g/100g bw respectively (Table 12).

4.15. EFFECT OF COLD STRESS ON ADRENAL HISTOLOGY

In Group I (untreated-NCST) cockerels the adrenal histology showed the intermixing of adrenal cortex and medulla with no distinct regional distribution (Plate4. Fig. A). The Group II (GB+IGN treated-NCST) cockerels showed the Zona Fasciculata (ZF) and medulla (M) densely packed with cells (Plate 4. Fig. B). The adrenals from Group VII and IX (untreated-CST) cockerels revealed ZF thickly packed with cortical cells and with moderate number of lipid droplets while intracortical capillaries were constricted (Plate 8.Fig.A), where as the GB+IGN treated-CST cockerels showed ZF with less fibrosis and moderate number of lipid droplets (Plate 8. Fig.B).

4.16. EFFECT OF COLD STRESS ON ANTIBODY FORMING CELLS

4.16.1. Jerne's plaque assay

On day 7 after immunization with rat red blood cells (RRBC) Group I (untreated-NCST i.e., normal) cockerels exhibited a maximum of 4.99×10^6 plaque forming cells (PFC)/7.5×10^6 spleenocytes, which corresponds to 66.53 per cent spleenocytes exhibiting plaque forming capability (Fig. 13. and Table.13). Compared to all other groups the Group II (GB+IGN treated-NCST i.e., control

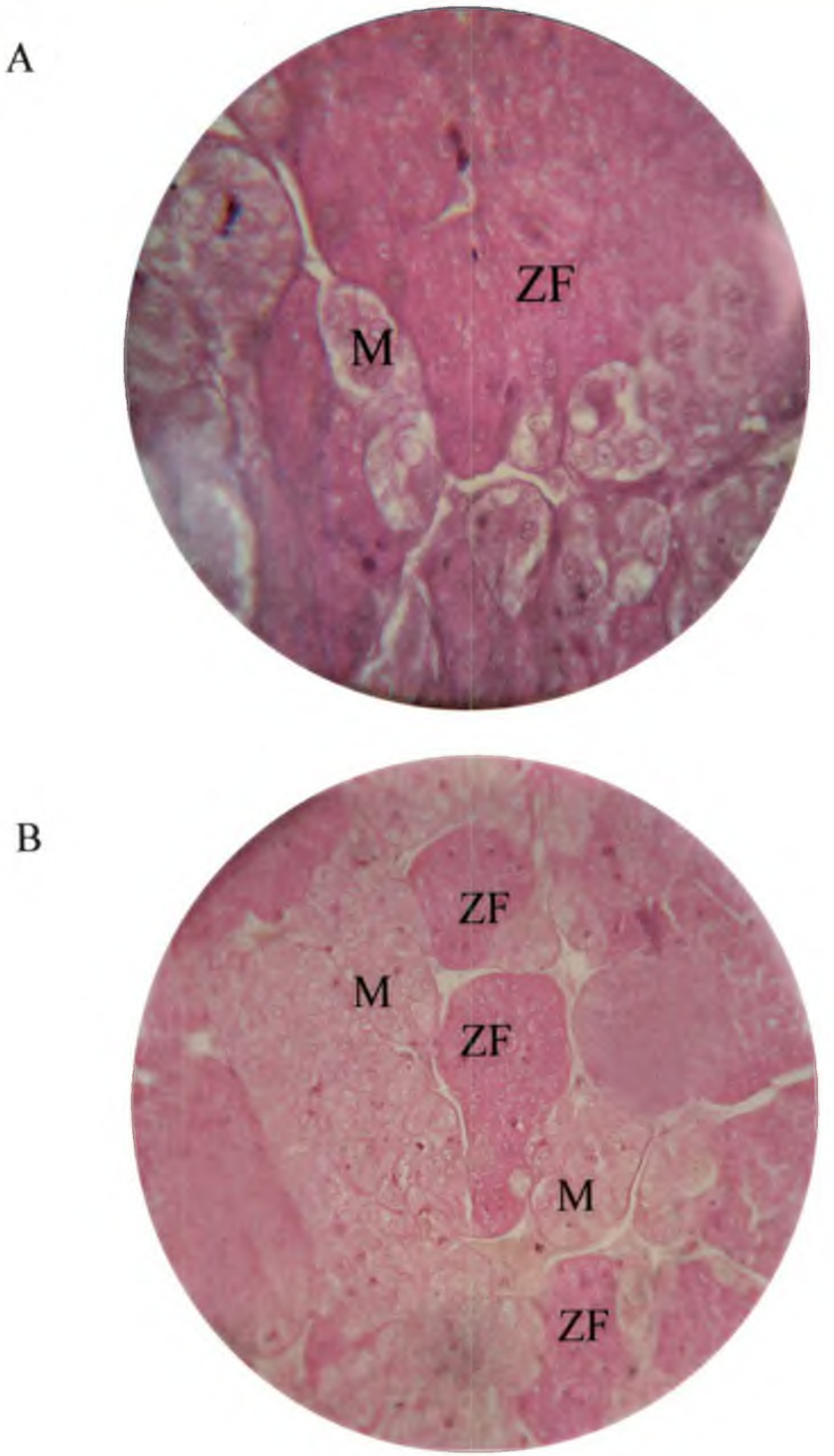


Plate 8. Effect of cold stress on adrenal histology (400X)

A. Adrenals of untreated, cold stressed cockerels

B. Adrenals of (GB+IGN) treated, cold stressed cockerels

ZF- Zona Fasciculata M- Medulla

(GB+IGN)) cockerels responded maximally by 84.00% ($6.3x10^6/7.5x10^6$) spleenocytes exhibiting plaque forming capability on 7th day (Plate 6). The Group V (untreated-CST i.e., cold alone) and Group VI (GB+IGN treated-CST i.e., cold + (GB+IGN)) cockerels exhibited a peak plaque forming response on 5th day and from day 7 onwards untreated-CST (cold alone) cockerels showed comparatively lower PFC response.

4.16.2. Rosette- forming cell (RFC) immunocytoadherence assay

On day 7 after immunization with rat red blood cells (RRBC) Group I (untreated- NCST i.e., normal) cockerels exhibited a maximum of $4.8x10^6$ RFC/ $7.5x10^6$ spleenocytes, and it corresponds to 64 per cent spleenocytes exhibiting rosette forming capability (Fig. 14 and Table14). Compared to all other groups the Group II (GB+IGN treated-NCST i.e., control (GB+IGN)) cockerels responded maximally by 89.20% ($6.69x10^6/7.5x10^6$) spleenocytes exhibiting rosette-forming capability on 7th day (Plate 7.Fig.A). Similar response was exhibited by Group V (untreated-CST i.e., cold alone) and Group VI (GB+IGN treated-CST i.e., cold + (GB+IGN)) cockerels, which showed a peak rosette forming response on 5th day. The untreated-CST (cold alone) cockerels showed comparatively lower RFC response through out the experiment (Plate 7.Fig.B).

Fig.13.Effect of cold stress on spleenic plaque forming cells (PFC)

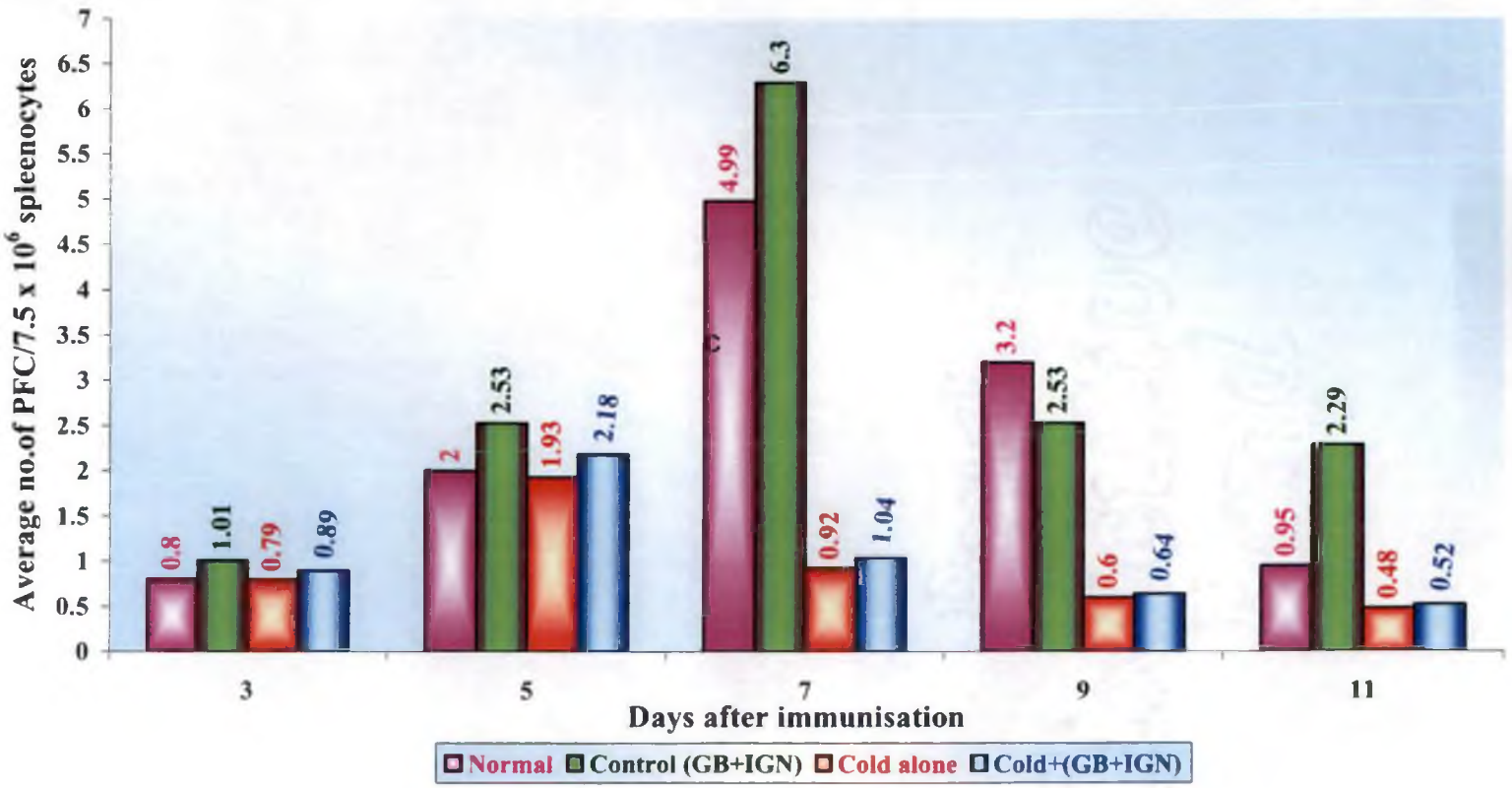


Fig.14.Effect of cold stress on rosette forming cells(RFC)

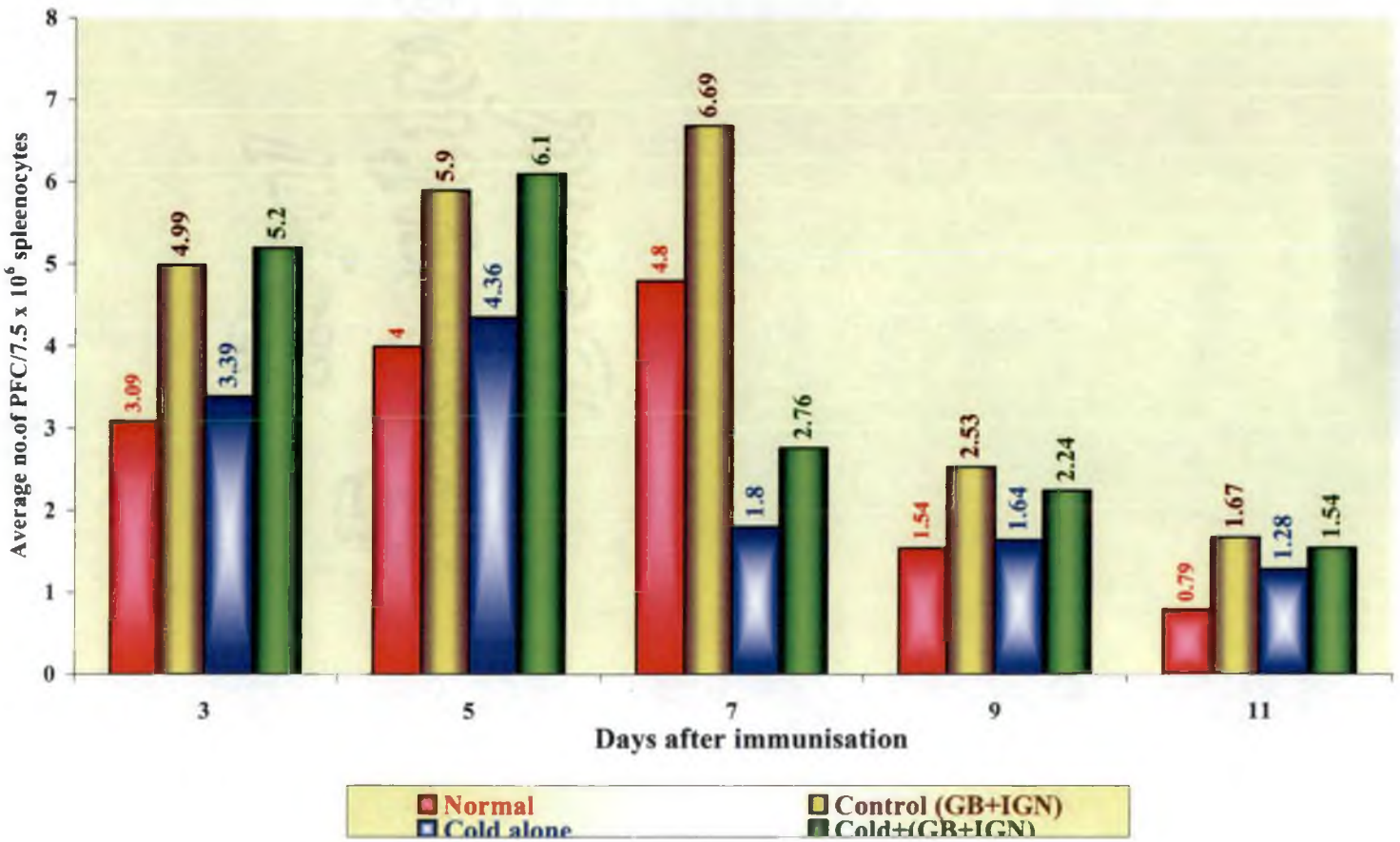


Table 13.Effect of cold stress on spleenic plaque forming cells (PFC) (n= 5)

Groups	Days after immunization with Rat red blood cells (RRBC)				
	3	5	7	9	11
I	10.67%	26.67%	66.53%	42.67%	12.67%
II	13.47%	33.73%	84.00%	33.73%	30.53%
V	10.53%	25.73%	12.30%	8.00%	6.40%
VI	11.87%	29.10%	13.87%	8.53%	6.93%

Group I – Normal (Untreated and non- cold stressed control)

Group II – Control (GB+IGN) (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.

Group V– Cold alone (Untreated and cold stressed (8±1°C, RH 40-50% for 4 h/day)

Group VI– Cold +(GB+IGN) (GB+IGN) treated and cold stressed.

Table.14.Effect of cold stress on rosette forming cells (RFC) (n= 5)

Groups	Days after immunization with Rat red blood cells (RRBC)				
	3	5	7	9	11
I	41.20%	53.30%	64.00%	20.50%	10.53%
II	66.53%	78.66%	89.20%	33.73%	22.27%
V	45.20%	58.13%	24.00%	21.87%	17.06%
VI	69.33%	81.33%	36.80%	29.86%	20.27%

Group I – Normal (Untreated and non- cold stressed control)

Group II – Control (GB+IGN) (GB+IGN) treated (2 g/kg bw for 20 days orally) and non- cold stressed control.

Group V– Cold alone (Untreated and cold stressed (8±1°C, RH 40-50% for 4 h/day)

Group VI– Cold +(GB+IGN) (GB+IGN) treated and cold stressed.

Discussion

5. DISCUSSION

Numerous environmental factors evoke a set of functional and morphological changes, called stress in living organisms. These changes most often lead to an adaptation, making it possible for the organism to survive in the challenging conditions. In birds however, it is difficult to specify a typical stress picture and predict the course of stress, it will be useful to learn how stressors affect the immune system of birds, and the adaptive capacity to respond, and the time it takes to return to homeostasis.

5.1. EFFECT OF HEAT STRESS ON BODY TEMPERATURE

The present study revealed that the rectal temperature of both untreated and GB+IGN treated cockerels exposed to heat stress resulted in rise of body temperature to 44.00 ± 0.20°C while NHST cockerels recorded mean rectal temperature of 42.90±0.20°C. This finding agreed with the reports of Yahav *et al.* (1997) where acute exposure of chickens to high temperature resulted in hyperthermia. The temporary or passive hyperthermia could also be due to accelerated muscular work associated with increased respiratory and cardiovascular activity as well as increased metabolic heat production as reported by Siegel (1980).

Birds are non-sweating animals; normally regulate the body temperature by vasomotor control of the surface blood supply especially through featherless areas of the skin surface like comb and wattles and by vascular convection. In the present study cockerels exhibited diarrhea and a great deal of behavioral changes such as gular flutter (panting), drooping of wings, prostration and drowsiness during the period of heat stress. The involuntary erector plumorum muscles, which produce the movement of individual feathers as well as voluntary cutaneous muscles for generalized skin movement facilitating cooling of the body, are important in heat exchange. 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 study also revealed that those cockerels exposed to heat, lost an average 3.4% of body weight. This findings agreed with the recordings of Bartholomew *et al.* (1954) and McKee *et al.* (1997) as they stated that in hot environments, the rate of water loss (evaporative and excretory) exceeds the production of metabolic water leading to loss of body weight and survival thereafter depends on availability of water. Rao *et al.* (2005) reported that oral administration of GB resulted in slight increase in body weight in rats. Though NHST cockerels treated with GB+IGN did not show any variation in the body weight, the treatment with GB+IGN during HST helped to maintain the body weight during the experiment.

The breed Gramapriya being evolved from White Leghorns were able to withstand higher temperature, as White Leghorns have been proved to withstand high temperature because of their willingness to drink large amounts of water (Fox, 1951) and possess a better evaporating cooling mechanism (Ota and McNally, 1961).

5.2.EFFECT OF HEAT STRESS ON HAEMATOLOGICAL PARAMETERS

The haematological parameters are important in the assessment of health status and disease of birds (Hawkey *et al.,* 1983) and are also good indices of livestock adaptability to prevailing environmental conditions (Kaushish *et al.* 1976).

5.2.1. Haemoglobin concentration (Hb)

During heat stress, there was a significant increase in Hb content in untreated-5 days HST cockerels and later the Hb content decreased to reach the basal line as in 10 days HST cockerels and that of untreated-NHST cockerels. This agreed with the findings of Luger *et al.* (2003) where the development of mature peripheral red blood cells from pluripotent stem cells in the bone marrow as a complex process regulated by erythropoietin, corticosterone, and triiodothyronine (T_3) and growth factors.

The glucocorticoid hormone is a key regulator for self renewal of erythroid progenitors (Wessely *et al.*, 1997), whereas (T_3) mainly controls the cell differentiation pathway, with strong haemoglobin accumulation (Bauer *et al.*, 1998). During acute heat stress (5 days HST) due to these factors, there might have been an increased erythropoeisis resulting in increased Hb concentration. Later on, during chronic heat exposure (10 days HST) as a measure of adaptogenesity (Koko *et al.*, 2004), cockerels would have consumed more water and thus prevented any significant change in Hb concentration, on account of haemoconcentration.

The present investigation revealed that both untreated and GB+IGN treated-NHST cockerels did not differ in Hb concentration. Pawar and Somkumar (2001) reported that GB administration resulted in an increased Hb concentration by 15.89% in rats. However, Gowri *et al.* (2001) explained that tannin content is inversely related with the haematinic effect of GB, as it reduces the bioavailability of iron. IGN contains 32% tannin (Parmar and Kaushal, 1982) and this could have opposed the haematinic effect of GB in the drug.

The present study also revealed that GB+IGN could not elicit any significant effect in Hb concentration during heat stress in cockerels as the birds treated with GB+IGN subjected to 5 and 10 days heat stress exhibited similar response as that of its untreated counter parts.

5.2.2. Packed cell volume (PCV)

The untreated-5 days HST cockerels showed a significant increase in PCV when compared with the untreated-NHST cockerels. Elevation in PCV could be caused by diminished plasma volume due to a mild dehydration, leading to haemoconcentration in hyperthermia as reported by Yahav and Hurwitz (1996). The value of PCV is a good indicator of the haemogram, and especially of the number of circulating erythrocytes and haemoglobin (Benjamin, 1985). Changes in PCV are probably related to modulation of the supply of oxygen to accommodate changes in heat production (Yahav *et al.*, 1997). Present findings

also agreed with the findings of Wessely *et al.* (1997) as they stated that alternations in the corticosterone concentration during stress was linearly correlated to PCV and may partially account for the defective erythropoeisis regulation that lead to accelerated proliferation of erythroid progenitors with impaired differentiation, resulting in the accumulation of immature erythrocytes in the blood stream. While in untreated-10 days HST cockerels, the PCV value decreased significantly and reached the basal value as that of untreated NHST cockerels, which referred to the ability of the birds to adjust to environmental variability, extremes, and cope with the consequences of changes associated with stress as explained by Hangalapura *et al.* (2004).

The investigation also revealed that both untreated and GB+IGN treated-NHST cockerels did not differ in PCV content. However, Pawar and Somkumar (2001) reported that GB administration resulted in increased PCV by 18.89% in rats. The absence of haematinic activity could be attributed to the inhibitory property of tannin present in IGN (Gowri *et al.*, 2001).

In the present study GB+IGN treated-5 days HST cockerels showed a non significant increase in PCV and it might be due to mild dehydration, and the increased erythropoeisis. Later, it decreased significantly to reach the PCV value as that of GB+IGN treated-NHST cockerels. Both untreated and GB+IGN treated cockerels responded similarly, but the birds treated with drug exhibited a great deal of capacity to return to homeostasis on chronic heat exposure for 10 days.

5.2.3. Total leucocyte count (TLC)

The result of the present study revealed that untreated cockerels exposed to 5 days HST exhibited a significant reduction in TLC when compared with untreated- NHST cockerels. Gross and Siegel, (1983) reported that changes in chicken leucocytes, in response to stress to be less variable and thus acts as a reliable indicator. The prolonged activation of hypothalamo-hypophyseal–adrenal (HPA) axis during heat stress was responsible for increased corticosteroid release, which results in immunosuppression and related leucocytopenia as

reported by Graczyk *et al.* (2003b). But the TLC value increased significantly on chronic heat exposure (10 days HST), which may be due to the innate mechanism of thermotolerance (Hangalapura *et al.*, 2004).

The GB+IGN treated-NHST cockerels showed a non significant increase in TLC when compared to untreated-NHST cockerels. Rekha *et al.* (1998) reported that oral administration of Brahma Rasayana that containing GB and IGN as major components, significantly increased TLC count in mice. Harikumar *et al.* (2004) also reported a significantly increased TLC in GB treated rats. Though a significant reduction was noticed in TLC count in GB+IGN treated-5 days HST cockerels, the value increased significantly in GB+IGN treated-10 days HST cockerels. This variation was also significantly over the untreated-5 and 10 days HST cockerels. This finding reinforced the immunomodulatory property as well as the leucocyte proliferative capability of the drug. This finding also agreed with reports of Sairam *et al.* (2002) where GB had an emphasized role in immuno compromised states, through its lymphocyte proliferative property. Srikumar *et al.* (2005) reported that administration of Triphala containing GB, IGN and *Terminalia belerica* significantly increased TLC in stressed rats.

From the present study, it was confirmed that GB+IGN has mitogenic property on stem cells of leucocytes during acute and chronic heat stress.

5.2.4. Lymphocyte (L) count

The result of the present study revealed that untreated cockerels exposed to 5 days HST exhibited a significant reduction in lymphocyte count when compared with untreated, NHST cockerels. Graczyk *et al.* (2003b) reported that when broilers are subjected to stress, involution of lymphoid organs occurred due to continued activation of HPA axis and release of adrenal steroids, resulting in lymphocytopenia. The findings of this study closely agreed with findings of Ben Nathan *et al.* (1976); Maxwell *et al.* (1990); and Borges *et al.* (2003) where significant decrease in circulating lymphocyte count was observed during stress in

chicks. The reduction in the absolute lymphocyte count or the lymphocytopenia identified in this study led to leucocytopenia in the corresponding groups.

Pardue and Thaxton (1984) reported that ascorbic acid (AA) enhances lymphocyte production and lymphocyte transformation, otherwise called as immunoenhancement property. Scartezzini *et al.* (2006) reported that on Ayurvedic method of processing there was an increase of the AA content of GB to 1.28% $^{w}/_{w}$. The result of the present study agreed with study of Sairam *et al.* (2002) and Gandhi and Nair (2005) where GB and IGN were reported to have an emphasized role in immuno compromised states, through its lymphocyte proliferative property.

5.2.5.Heterophil (H) count

The study indicated that untreated-5 days HST cockerels exhibited a significant increase in heterophil count when compared with untreated-NHST cockerels and this findings agreed with the observation of Borges *et al.* (2003) where significant increase in heterophil count was observed during heat stress in male broiler chicks. This finding also agreed with the reports of Altan *et al.* (2000) as they correlated increased rectal temperature with an increase in heterophil proportion in the circulating blood. Ader and Cohen (1993) stated that increased corticosterone levels would suppress neutrophil function and increase the number of neutrophils in stressed rats. Results also indicated that upon chronic exposure to heat (10 days HST) the untreated birds exhibited a significant reduction in heterophil count. This could be attributed to the adaptive nature of animals or birds upon exposure to extreme environmental change for prolonged period as suggested by Hangalapura *et al.* (2004).

The GB+IGN treated-NHST cockerels showed a non significant increase in heterophil count when compared with untreated, NHST cockerels. This agreed with the findings of Srikumar *et al.* (2005) who stated that treatment with GB, IGN, and *Terminalia belerica* (Triphala) could stimulate the neutrophil

function in rats. The GB+IGN treated-5 days HST cockerels exhibited a similar response in heterophil count as the untreated-5days HST cockerels. In GB+IGN treated-10 days HST cockerels, the heterophil count decreased non significantly, as that of its untreated counterparts.

The results of the present study thus revealed that upon prolonged treatment with GB+IGN the tendency to restore to normal homeostasis could be promoted.

5.2.6.Heterophil/ Lymphocyte (H/L) ratio

The results of the present study showed that untreated-5 days HST cockerels exhibited a significantly higher H/L ratio when compared with untreated-NHST cockerels and this observation was in close agreement with earlier reports of Jones (1989). The H/L ratio has been indicated to be a good quantitative measure of stress. (Zulkifli and Siegel, 1995). The H/L ratio had been showed to be highly heritable (Altan *et al.*, 2000) and a reliable index for determining stress in poultry (Gross *et al.*, 1985). Borges (2003) reported increase in heterophils and decrease in lymphocytes occurs during stress in chicks. In untreated-10 days HST cockerels there was a significant reduction in the H/L ratio and this may be attributed to the adaptation of the birds to prolonged heat exposure as suggested by Katanbef *et al.* (1988); Maxwell *et al.* (1990), and Zuidhof *et al.* (1995).

In GB+IGN treated-NHST cockerels the H/L ratio was slightly higher than that of untreated-NHST cockerels. GB+IGN treated-5 days HST cockerels exhibited a significantly higher value, and then the ratio decreased significantly by 10 days of heat exposure. The ratio noticed in GB+IGN treated-5 and 10 days HST cockerels was significantly lower than that in untreated 5 and 10 days HST cockerels. In the GB+IGN treated-10 days HST cockerels the value was significantly lower than that in the drug treated-5 days HST cockerels.

Thus it could be concluded that GB+IGN treatment could enhance the restoration of normal homeostasis.

5.3. EFFECT OF HEAT STRESS ON SOME BIOCHEMICAL PARAMETERS

In birds, some physiological and biochemical adaptations could occur to protect essential cell functions against heat stress and to permit a rapid recovery from mild or even moderate hyperthermic damage. Each tissue and organ differs in its sensitivity for sustaining thermal injury and in the rate of recovery processes. Therefore, it is necessary to study the biochemical features of hyperthermic damage under hot environmental conditions and the restorative mechanism during the stressful period.

5.3.1. Serum protein profile:

5.3.1.1. *Serum total protein content*

The results of the study revealed that serum total protein content decreased non significantly in 5 days HST cockerels when compared with untreated-NHST counterparts. Later on, untreated cockerels when subjected to 10 days HST exhibited a non significant increase. Stress has been reported to increase protein catabolism (Brown *et al.*, 1958; Negra and Meyer, 1963). Nazifi *et al.* (2003) stated that total protein content was less affected in heat than in cold stress. Beard (1987) explained that during stress, body proteins are broken down and amino acids are shunted away from growth and are used by specific cells to synthesis critical proteins like acute phase proteins and antibodies which allow the bird to mount a successful immune response and adapt. One of the deleterious stress responses in poultry is significant reduction in total protein level because of the increased corticosteroid secretion resulting in increased protein derived gluconeogenesis (Burger and Denver, 2002; Debut *et al.*, 2005)

The GB+IGN treated-NHST cockerels exhibited slightly higher total protein than the untreated-NHST cockerels. This may be attributed to the

immunomodulatary effect of GB+IGN and thereby preventing the protein catabolism (Srikumar *et al.*, 2005). The GB+IGN treated-5 days HST cockerels showed a non significant decrease and when cockerels are treated and exposed to heat for 10 days, their total protein value slightly increased. Iheukwumere and Herbert (2003) reported a non significant reduction of total protein in dehydrated broiler chickens.

Though the changes in the total protein were not prominent, the result of present study enlightened the effect of GB+IGN in preventing the protein catabolism during stress.

5.3.1.2. *Albumin content*

The results of the study showed that untreated-5 days HST cockerels exhibited a non significant decrease in albumin concentration when compared with untreated-NHST cockerels and this may be due to impaired protein synthesis during acute stress (Beard, 1987) and because of catabolism of the proteins for the production of critical proteins. Albumin is being considered to be most assessable substrate for gluconeogenesis by liver (Pardue *et al.*, 1985). Later on, with chronic stress for 10 days, untreated cockerels showed a non significant increase in the albumin concentration.

The GB+IGN treated-NHST cockerels exhibited a non significant increase in their albumin content when compared with its untreated-NHST cockerels. This agreed with the findings of Pardue *et al.* (1985) who stated that heat stressed birds receiving AA had lower corticosterone levels and therefore exhibited a significant reduction in the protein derived gluconeogenesis. The GB+IGN treated-5 days HST cockerels exhibited a non significant reduction in the albumin content, which then increased non significantly to reach almost the same value as shown by treated-NHST cockerels. This finding reinforced the capability of the drug to prevent protein catabolism (Srikumar *et al.*, 2005).

Though, the changes encountered were non significant, the study indicated that prolonged treatment with GB+IGN resulted in a greater tendency to reach the basal line albumin concentration during chronic exposure to heat stress.

5.3.1.3. *Globulin content*

In the present study, when the untreated-5 days HST cockerels were compared with the untreated-NHST cockerels, there was non significant variation noticed in globulin concentration, but the value remained almost unchanged in 10 days HST cockerels. This finding agreed with Thaxton (1978) who stated that an increased synthesis of immunoglobulins might have occurred and could reflected an altered protein metabolism, which favours globulin synthesis in birds exposed to stress.

The GB + 1GN treated-NHST cockerels showed slightly higher value than that of its untreated counterparts. The drug treated-5 days HST cockerels showed a non significant decrease, while in the treated-10 days cockerels the globulin content remained almost unchanged. This indicated that globulin fraction was not much influenced either by acute or chronic heat stress or by GB+IGN treatment.

5.3.2. Serum enzyme activities

5.3.2.1. *Serum lactate dehydrogenase (LDH) activity*

The results of present study pointed out that untreated-5 days HST cockerels had a significantly higher value of LDH than the NHST cockerels, which further increased non significantly in untreated-10 days HST cockerels. LDH isoenzymes had been found in most avian tissues and a significant increase in LDH activity represented an overall damage to the system (Kendel, 2002). Van der Linde *et al.* (1992) examined the role of LDH isoenzymes in heat exposed rats, and concluded that the isoenzymes could be useful diagnostically and prognostically in heat stress. Mujahid *et al.* (2006) explained that acute heat stress resulted in increased levels of reactive oxygen species in mitochondria

isolated from the skeletal muscle of broilers and an increased superoxide production in skeletal (pectoralis) muscle, which could lead to more leakage of LDH into the circulation.

The GB + 1GN supplementation had significantly reduced the leakage of LDH from various tissues during the period of heat stress especially in 5 days-HST cockerels when compared to untreated HST groups. This could be attributed to the cytoprotective effect GB as explained by Sairam *et al.* (2002) and that of IGN as reported by Lee *et al.* (2005) as they established that extract from fruits of IGN significantly reversed the hepatotoxicity and LDH leakage into circulation from tissues, owing to the stabilizing capacity of the drug on the cell membrane permeability. Similar results were reported by Naik *et al.* (2004), and Suchalatha and Shyamaladevi (2004) as they demonstrated the cytoprotective action of IGN in rats.

The GB+IGN treated-5 days HST cockerels showed significantly lower values of LDH than their untreated counterparts. Hence, it was concluded that GB+IGN treatment has got protective action during acute heat stress. While no significant variation was observed in LDH concentration between GB+IGN treated and untreated 10 days HST cockerels, it can be concluded that the drug treatment was useful during acute stress than in chronic stress.

5.3.2.2. *Serum creatine kinase (CK) activity*

The untreated-NHST cockerels exhibited significantly higher CK activity than that of GB+IGN treated-NHST cockerels. The untreated-5 and 10 days HST cockerels exhibited significantly higher value than that of GB+IGN treated counterparts. CK is mainly found in all muscles and brain tissues and it plays an important role in energy storing mechanism of the tissues (Kew *et al.*, 1971). Elevations in serum CK activities in birds are indicative of skeletal muscle damage or myopathy, consequent to the disruption of sarcolemma function and altered permeability (Mitchell and Sandercock, 1995). Serum CK activity also depended on muscle cell metabolism and turn over especially in broiler birds

(Sandercock *et al.*, 2001). Debut *et al.* (2005) reported that during heat stress CK activity was enhanced especially in the breast (pectoralis major) and thigh (ilio tibialis) muscles in chickens. Lin *et al.* (2006) also reported a significantly higher CK activity in broiler chickens exposed to high temperature.

CK is also associated with hypokalemia as Rubel and Ishak, (1983) stated that during heat stress the initial hyperkalmia due to excessive diarrhoea, one of the common features in birds. Further more, high levels of rennin activity occur because of fluid depletion (Knochel, 1978) and excessive production of aldosterone to re-establish osmotic homeostasis (Koko *et al.* 2004), which leads to further conservation of sodium and depletion of potassium. Hypokalemia leads to increased cellular permeability and loss of muscle cell membrane permeability and loss of muscle cell membrane integrity, which will subsequently lead to increased release of CK. Terblanche and Nel (1998) reported that the acclimation resulted in significantly lower CK activity levels in heat stressed rats. But such a decrease was not observed in this study, indicating that birds require further more days to get acclimatized.

The GB + 1GN supplementation had significantly reduced the leakage of CK from various tissues during the period of heat stress in 5 days HST cockerels when compared to untreated HST groups. This could be attributed to the cytoprotective effect of the drug, owing to the stabilizing capacity on the cell membrane permeability as explained by Sairam *et al.* (2002); Suchalatha and Shyamaladevi (2004) and Lee *et al.* (2005).

The GB+IGN treated-10 days HST cockerels showed significantly lower values of CK than their untreated counterparts. Hence, it was concluded that GB+IGN treatment has got protective action during acute as well as chronic heat stress period and it was also convinced that drug treatment for prolonged period during chronic stress played an important role in restoring the normal level of CK.

5.3.3. Serum electrolytes level

5.3.3.1.*Serum sodium (Na) level*

The results of the present study revealed a non significant increase in sodium (Na) concentration in acute heat stressed untreated (5days HST) cockerels. Debut *et al.* (2005) reported that acute heat stress affected the electrolytes level with an increase in Na concentration in broiler chickens. No significant reduction was noticed on chronic heat exposure in untreated cockerels in this study. This finding did not agree with Borges *et al.* (2003) as they reported that chronic heat stress decreased blood Na level in broilers.

The GB+IGN treated-NHST cockerels showed a non significant increase in Na concentration than that of untreated-NHST cockerels, and that value decreased slightly on heat exposure for 5 days in treated cockerels and further decreased non significantly in treated-10 days HST cockerels. These findings agreed with Mehmet *et al.* (2005) as they reported that blood Na level was not affected by dietary ascorbic acid supplementation in Japanese quails reared under hot conditions.

The present study revealed that GB+IGN treatment could not bring about any significant change in Na content during acute and chronic heat stress.

5.3.3.2. *Serum potassium (K) content*

The result of the present study indicated that potassium (K) concentration increased slightly in untreated-5 days HST cockerels. Khone and Jones (1975); Beker and Teeter (1994) reported an increase in blood K level in response to heat stress. A pronounced diarrhea that was observed during the study in HST cockerels could be one of the attributing factors for increased serum K level and Pardue *et al.* (1985) reported similar observations in chicks. While in untreated-cockerels the K level decreased significantly on 10 days heat exposure. This finding agreed with Borges *et al.* (2003) as they reported that K response seems to

be related to the time under heat stress because broilers subjected to cyclic periods of stress resulted in reduction of blood K level. Excess of K competes for buffer anions from the renal tubular fluid, preventing the removal of some of the H^+ thus preventing from getting reabsorbed and development of acidosis (Wilson, 2003). Further more, high levels of rennin activity occur because of fluid depletion and possibly excessive production of aldosterone to reestablish osmotic homeostasis (Koko *et al.*, 2004), which leads to further conservation of Na and depletion of K (Knochel, 1978).

The GB+IGN treated-NHST cockerels showed slightly higher value than untreated, NHST cockerels. Parmar and Kaushal (1982) explained that among the mineral content of edible portion of GB and IGN, K constitutes about 0.37 and 1.27 % respectively. This could also be the possible reason for the slight increase in K level of drug treated cockerels.

GB+IGN treated-5 days HST cockerels exhibited a similar response as that of its untreated counterparts indicating that the drug treatment could not elicit remarkable benefits during acute heat stress. While drug treated GB+IGN treated-10 days HST cockerels showed a non significant increase in the K level and those birds exhibited a great deal of tendency to restore the mineral homeostasis.

5.3.4. Serum C-reactive protein (CRP)

The results of the present study revealed that GB+IGN treated-NHST cockerels exhibited CRP value lower than that of untreated-NHST cockerels. CRPs are being considered as a positive acute phase protein (Gabay and Kushner, 1999). Kumar *et al.* (2005) stated that CRPs are prototype acute phase reactant in birds, and its plasma level increases as much as 1000 fold especially during cellular injuries and trauma associated with infection or inflammation and stress related physiological changes.

The untreated cockerels when exposed to heat exhibited higher value than that of GB+IGN treated counterparts. The present study showed that GB+IGN

treatment in NHST cockerels could bring about a slight decrease in CRP level which reinforced the cytoprotective effect of the GB+IGN as there was only moderate increase in circulating CRP levels during heat stress in GB+IGN treated cockerels, when compared to untreated-HST cockerels. And these findings agreed the cytoprotective property of GB and IGN as reported by Sairam (2003) and Lee *et al.* (2005).

5.4. EFFECT OF HEAT STRESS ON CERTAIN SERUM HORMONES LEVEL

5.4.1. Serum cortisol concentration

The present study revealed a significant increase in serum cortisol level in untreated-5 days HST cockerels when compared with untreated-NHST cockerels. Dallman *et al.* (1992) suggested that major neuroendocrine mechanism in heat stress reaction was the activation of the HPA axis. Corticotropin releasing factor (CRF) was released from the median eminence of the hypothalamus in response to stress, which stimulates the synthesis of adrenocorticotropin hormone (ACTH) (Krieger, 1983). Peripheral lymphocytes also respond to heat stress with increased secretion of ACTH (Stefano and Smith, 1996). De Nicola *et al.* (1968) reported that ACTH produced during stress inhibited AA transport in to the rat adrenal, resulting in significantly increased in steroidogenesis.

ACTH was then transported into the circulation and inhibits AA transport into adrenals and correlated with this inhibition was a concomitant increase in steirodogenesis (Negra *et al.*, 1962). Even though corticosterone is the major steroid in poultry, cortisol also acts as a useful indicator of short term stress (Grandin, 1997) and it takes about 10 to 20 min to reach the peak level. After the stressors were withdrawn, the concentration dramatically declined to the normal level probably due to the short half life (1- 1 $^{1}/_{2}$ h) of cortisol (Stull and Rodiek, 2000).

The untreated-5 days HST cockerels showed significantly higher cortisol value when compared with that of 10 days HST cockerels and this indicated

increased steroid synthesis during acute heat stress. This finding agreed with reports of Lin *et al.* (2006) as they noticed an increased 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.

Gooseberry (GB) is considered to be robust source of AA (Vit. C) among the available herbs worldwide (Khandelwal *et al.*, 2002). Numerous studies suggested that AA functions as a regulator of adrenal steroids because high levels of AA had been found to inhibit steroidogenesis (Kitabchi, 1967). AA has been shown to inhibit the enzymatic side chain cleavage system, which converts cholesterol to pregnenolone (Shimizu, 1970; Carballeira *et al.*, 1974). AA has also showed to inhibit C-21 hydroxylase and 11-β hydroxylase in steroidogenic pathways (Cooper and Rosenthal, 1962). It was also reported that urinary excretion of water soluble AA had been shown to increase along with increased cation excretion (Pardue *et al.*, 1985) in AA supplemented chicks exposed to high environmental temperature.

While GB+IGN treated-5 and 10 days HST cockerels exhibited significantly lower value than its untreated counter parts, drug treated birds exposed to chronic heat stress showed a great deal of tendency to return to homeostasis. This agreed with findings of Pardue and Thaxton (1984) as they stated a similar response in chicks fed with AA. Dietary AA may inhibit steroidogenesis by elevating adrenal AA, thus limiting some of the deleterious responses associated with stress and delay the depletion of steroid hormone precursors. Hence the amelioration of these heat induced effects may be attributed to properties of GB+IGN either by reducing glucocorticoids output and/or protection of cells from the cytotoxic effects of adrenal steroids. This finding also coincided with reports of Srikumar *et al.* (2005) they explained that treatment with GB, IGN and *T. belerica* (Triphala) resulted in decreased corticosterone level in rats. Therefore it can be concluded that as a measure of adaptation by overcoming the increased elimination rate of ascorbate and decreased transport

into adrenals under ACTH coverage, GB+IGN supplemented HST birds could have attained enough AA in the adrenals on prolonged drug treatment.

5.4.2. Serum triiodothyronine (T_3) concentration

The results of the present study showed a significant increase in T_3 level in untreated-5 days HST cockerels when compared with untreated-NHST cockerels and a further significant decrease was noticed in 10 days NHST cockerels. These findings positively correlated with the reports of Mitchell and Sandercock (1995) and Rudas and Pethas (1984), as they found that the increased production of growth hormone on exposure to heat was the causative factor for an increase T_3 in blood mediated via stimulation for conversion of T_4 to T_3. Thyroid hormones have been involved in thermoregulation in birds. Lin *et al.* (2006) also reported a significant increase in T_3 level when broilers are exposed to acute heat stress. A hypermetabolic response is counter to homeostatic mechanisms to regulate core temperature during heat stress (Gordon *et al.*, 2000). Changes in the thyroid function in response to environmental stimuli were complex that probably involves changes in thyroid gland output, peripheral conversion of T_4 to T_3, degradation of T_3 and regulation of cellular receptors for thyroid hormones as suggested by Darras *et al.* (1995). Collin *et al.* (2005) demonstrated that certain avian uncoupling proteins (aUCPs) are involved in mitochondrial proton electrochemical gradient that would uncouple phosphorylation from oxidative enzymes in birds, and hence produce heat from muscle and adipose tissue. The over production of aUCP be involved with high T_3 concentrations and thermogenesis in stress conditions.

GB+IGN treated-NHST and HST cockerels exhibited significantly lower T_3 levels than its untreated counterparts. This may be attributed to the activity of the drug and coincided with the findings of Panda *et al.* (2002) where the role of GB in ameliorating hyperthyroidism by decreasing the thyroid hormone concentration in mice and with Parmar *et al.* (2006) they explained the effect of *Terminalia arjuna* extract in decreasing the level of thyroid hormones. These

findings thus revealed the effect of prolonged treatment of GB and IGN in reducing the T_3 level and to reestablish the normal level in heat stressed cockerels.

5.4.3. Serum thyroxine (T_4) concentration

The results of the present study showed a non significant increase in T_4 level in untreated-5 days HST cockerels when compared with untreated-NHST cockerels. The untreated-5 days HST cockerels showed significant reduction in the T_4 level when compared with untreated-10 days HST cockerels. These findings partly coincided with reports of Geraert *et al.* (1996) who claimed that plasma T_4 concentration did not change in broiler chickens under hot conditions, as because deiodinase activity was made greatly reduced leading to the conservation of T_4 level.

The GB+IGN treated-5 days HST cockerels showed a significant reduction of T_4 level when compared with the drug treated-NHST cockerels. In GB+IGN treated-10 days HST cockerels T_4 level returned to basal line in par with controls. This may be attributed to the activity of the drug and coincided with the findings of Panda *et al.* (2002) as they explained the role of GB in ameliorating hyperthyroidism by decreasing the thyroid hormone concentration in mice and with Parmar *et al.* (2006), they explained the effect of *Terminalia arjuna* extract in decreasing the level of thyroid hormones. GB+IGN treated-10 days HST cockerels did not show variation with that untreated-10 days HST cockerels and thus it could be concluded that the drug reduces T_4 level during acute heat stress while no such beneficial effect was noticed during chronic heat stress.

5.5. ELECTROPHORETIC SEPERATION OF SERUM PROTEINS

The pattern of electrophoretic separation of serum proteins obtained in the study revealed that under reducing conditions untreated-HST cockerels (both 5 and 10 days) showed missing of certain high molecular weight serum proteins with a concomitant appearance of new low molecular weight protein bands. Such

an observation was not encountered with GB+IGN treated-HST cockerels, which showed almost similar pattern of separation with that of control cockerels. However, further detailed study has to be carried out in this regard to identify and characterize the nature of new proteins.

5.6. EFFECT OF HEAT STRESS ON IMMUNOLOGICAL PARAMETRERS

Environmental temperature has an influence on the immune response of poultry (Beard and Michell, 1987). The effect of environmental temperature on immune responsiveness might depend on the demand that was made on the bird's capacities to maintain homeothermia (Henken *et al.*, 1982). As per the reports of Beisel (1977) during acute heat stress and under the influence of glucocorticoids, body proteins were broken down, amino acids were shunted away from growth and are used by specific cells to synthesize critical proteins which allow the bird to mount a successful immune response to a particular antigen challenge. The role of antistress agents like AA play an important role at this juncture as they stimulate lymphoid cells to increase in number (Pardue and Thaxton, 1984; Bains, 1996; Ansari *et al.*, 1998), and acute phase proteins as well as antibodies also to increase through the use of amino acids.

5.6.1. Haemaglutinin (HA) assay

The results of present study indicated that anti SRBC HA titre exhibited by GB+IGN treated-HST and NHST cockerels were comparatively higher than those of untreated- HST and NHST cockerels. The results also agreed with the earlier reports of Subba Rao and Glick (1970) where heating episodes limit humoral immune potential in chicken against SRBC. Thaxton and Siegel (1970) established that preformed antibody levels could be reduced by higher environmental temperature. This effect was defined as high environment temperature mediated immunodepression possibly mediated through hypothalamo- hypophyseal-adrenal axis. The effect of environmental temperature on immune responsiveness may depend on the demand that was made upon the bird's capacities to maintain homeothermia (Henken *et al.*, 1982).

In spite of heat stress, GB+IGN treated cockerels showed a moderately persistent HA titre compared to untreated-HST cockerels. These findings positively correlated with the findings of Pardue *et al.* (1985) as they found that heating reduced HA titres and that heat mediated immunosuppression was ameliorated by AA supplementation. The AA can reduce synthesis and secretion of adrenal steroid hormones and it may protect immunobiologic tissues from steroid insult and also protects cell membranes, AA supplementation may alter biochemical mechanisms within lymphoid tissue (Moffat *et al.*, 1972) and these changes may enhance immune response and result in greater agglutinin production (Pardue and Thaxton, 1984). Thus, it was confirmed that during heat stress GB+IGN treatment protected the preformed antibodies from being catabolised and eliminated at an early date from the system. In other words, GB+IGN treatment during heat stress brought about an immunopotentiative effect on humoral immune response.

5.6.2. Haemolysin (HL) assay

The present study indicated that anti-SRBC HL titre exhibited by GB+IGN treated-HST and NHST cockerels were comparatively higher than those of untreated HST and NHST cockerels. The results of the present study also coincided with reports of Thaxton and Siegel (1970) as they established that preformed antibody levels could be reduced by higher environmental temperature. Even though HL assay has been considered as a rapid screening test for antibody titres it has got less sensitivity compared to HA assay (Subba Rao and Glick, 1970). The findings of present study indicated higher HL titre in GB+IGN treated cockerels, which reconfirmed the beneficial aspect of GB+IGN treatment in cockerels during heat stress.

5.6.3. Serum mercaptoethanol resistant antibody (Ig G) level

The present study indicated that the peak titre value of anti-SRBC IgG was obtained at an earlier date in GB+IGN treated-HST cockerels when compared to cockerels of untreated groups. It was also found that GB+IGN

treatment brought about a better anti-SRBC IgG titre development than untreated cockerels irrespective of heat stressed. The increased IgG titre encountered in GB+IGN treated cockerels could be attributed to either decreased rate of catabolism of antibodies (Srikumar *et al.*, 2005) or due to protective effect of GB+IGN supplementation on immune system of stressed cockerels.

5.6.4. Serum mercaptoethanol sensitive antibody (Ig M) level

The findings of the present study indicated that through GB+IGN treatment-HST cockerels developed a moderate titre of IgM against SRBC, which persisted at high level through out the heat exposure period, whereas in untreated HST cockerels there was a steep fall in the IgM titre value from the peak value during heat stress. However, the peak titre exhibited by HST cockerels was lower than that exhibited by NHST cockerels. These findings closely agreed with an earlier report by Thaxton (1978) as well as Subba Rao and Glick (1977) who reported that a hot environment favoured rapid catabolism of challenged antigen and a reduction in the level of antibody production.

5.7.EFFECT OF HEAT STRESS ON RELATIVE WEIGHT OF ADRENAL AND SPLEEN

5.7.1. The relative adrenal weight

The results of the present study showed that adrenals of untreated and GB+IGN treated-NHST cockerels did not differ in their relative weight. The untreated-5 days HST cockerels a non significant reduction while untreated-10 days HST cockerels showed a significant reduction in the adrenal weight and this finding agreed with the reports of Koko *et al.* (2004). GB+IGN treated-NHST cockerels did not differ in the relative weight of adrenals either by 5 or 10 days heat exposure. This finding of the present study agreed with the report of Pardue *et al.* (1985) who showed that hyperthermia did not affect adrenal weight in AA supplemented chicks.

5.7.2. The relative spleen weight

The present study revealed that GB+IGN treatment brought about a significant increase in relative spleen weight in 5 days HST cockerels compared to untreated HST cockerels. Stress had been coined with reduction in the weight of lymphoid organs such as spleen and bursa of Fabricius (Siegel, 1980). The increased spleenic weight encountered in the study on GB+IGN treated-NHST and 5 days HST cockerels could be attributed to the immunopotentiating action of AA resulting in appearance of new germinal centers in spleen (Graczyk *et al.*, 2003b). This observation reconfirmed an earlier report of Pardue *et al.* (1985) who found that AA supplementation in chicks resulted in larger bursa and spleen weight.

5.8. EFFECT OF HEAT STRESS ON ADRENAL HISTOLOGY

The histological section of adrenals of untreated NHST cockerels showed the intermixing of adrenal cortex and medulla with no distinct regional distribution and this confirmed with reports of coincided with Vestergaard and Willeberg, (1978) who explained the intermingling nature of cortical and medullary components constitutes a major characteristic of avian adrenal. The untreated and HST cockerels showed increased fibrosis and depletion of lipid droplets in Zona Fasciculata (ZF), and this observation agreed with Koko *et al.* (2004) as they explained that glucocorticoid synthesis is performed mostly in the ZF and heat exposure provoked a depletion of lipid droplets from the ZF cells. GB+IGN treated-NHST cockerels revealed ZF and medulla densely packed with cells and this could be attributed to the immunomodulatory property of the drug. However, only moderate level of fibrotic invasion could be observed in GB + 1GN treated HST cockerels, which reinforced the protective property of the drug during stress.

5.9. EFFECT OF HEAT STRESS ON ANTIBODY FORMING CELL

Haemolytic plaque forming cells (PFC) are abundant in the spleen but are less frequent in other lymphatic organs of immunized birds. Similarly, cells with immuno cytoadherant property (Rosette forming cells, RFC) appear in large number in the peripheral blood as well as in the spleen (Hirota *et al.* 1980; Seto and Henderson, 2005).

The screening of antibody tire was performed on the next day following antigen administration, PFC and RFC assays thus indicated that the kinetics of antibody response during the period of heat stress.

5.9.1. Jerne's plaque assay

The result of present study demonstrated that anti-RRBC PFCs in GB + IGN treated- NHST cockerels were significantly higher than untreated NHST controls. Similarly, treated-HST cockerels showed more percent of anti-RRBC PFCs compared to untreated-HST and NHST cockerels. This finding agreed with Fernandes (2000) and Fujiwara *et al.* (1999) they reported increased PFC during stress. Komori *et al.* (1996) revealed that during stress, a significant elevation of the plaque-forming cells (PFC) was found in spleen cells and that immune function was differentially affected by the duration of stress. Denno *et al.* (1994) also reported that alpha and beta receptor sites on lymphocytes for nor epinephrine and epinephrine were stimulated during stress and that these catecholamines have a regulatory role in plaque cell proliferation and thereby increasing PFC count.

The over all improved response showed by GB+IGN treated cockerels (both HST and NHST) compared to the untreated cockerels (both HST and NHST) could be attributed to the stimulative effect of the drug on the spleenic cells to produce antibodies against RRBC.

5.9.2. Rosette – forming cell (RFC) immunocyto adherence assay

The present study revealed that the number of anti-RRBC RFCs encountered were higher than number of anti-RRBC PFCs. This observation coincided with the earlier report of Tufveson and Alm (1975); Thursh *et al.* (1972) and Dagg *et al.* (1997). The result of present study demonstrated that anti SRBC RFCs in GB + IGN treated-NHST cockerels were significantly higher than untreated NHST controls. The study also confirmed that GB + IGN supplementation improved anti-RRBC RFCs in both NHST and HST cockerels when compared to untreated NHST and HST cockerels. Thus, it was reconfirmed that GB + IGN treatment stimulated spleenic cells with a capability to from rosettes or cytoadherence through the release into peripheral circulation.

The findings revealed that GB+IGN treatment was beneficial in antibody formation during heat stress.

5.10. EFFECT OF COLD STRESS ON BODY TEMPERATURE

The present study revealed that the rectal temperature of cockerels exposed to cold stress consecutively for 4h per day resulted in a narrow reduction in body temperature to 42.00 ± 0.20^{0}C while control birds recorded mean rectal temperature of 42.90±0.20^{0}C. Obrien *et al.* (1998) stated hypohyration as a predisposing factor for hypothermia and also contributed for the body weight loss by 4-5%. The findings of present study agreed with Thomas and George (1975) and Luger *et al.* (2002) where they reported that acute cold stress produced hypothermia in birds. Behaviorally, birds reduce heat loss by covering unfeathered portions of the body with feathered portions. In this study the cockerels exhibited a great deal of 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. Heat loss could be reduced 40-50% by sitting and about 12% by tucking the head under the wing (Deighton and Hutchinson, 1940). Huddling also reduces heat loss (Klieber and Winchestor, 1933). In cold environments birds fluff-out the feathers (Hutchinson, 1954),

thereby increasing the insulating layer of air, and conversely, tend to flatten the feathers close to body during hot periods.

The present study indicated that exposure of cockerels to cold environment, resulted in an average loss of 4% bw at the end of the stress period. 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 observations made by Dabbert *et al.* (1997) and Robbins (1983) where they suggested that exposure of birds to extreme cold could elevate existing metabolism three-fold over basal metabolic rate, and the body mass loss concomitant in cold stressed birds indicated that fat reserves were partly used to fuel these metabolic demands despite the availability of feed.

5.11.EFFECT OF COLD STRESS ON SOME HAEMATOLOGICAL PARAMETERS

5.11.1. Haemoglobin concentration (Hb)

During cold stress, there was a significant increase in Hb content in untreated-5 days CST and later it decreased in 10 days CST cockerels decreased to reach the basal line as that of untreated-NCST cockerels. The result of the present study also revealed that the Hb concentration of untreated and GB+IGN treated-NCST cockerels did not differ significantly. On cold exposure for 5 days without GB+IGN supplementation, the Hb concentration increased significantly and this may be attributed to a hypohydration that the cockerels encountered as indicated by 4.00% reduction during acute cold stress (Obrien *et al.*, 1998). Luger *et al.* (2003) explained that acute cold exposure leads to significant stress response, coupled with the failure to produce sufficient thyroid hormones, resulted in an enhanced erythropoeisis, which included the release of marked proportion of immature red blood cells. The glucocorticoid hormone is a key regulator for self renewal of erythroid progenitors (Wessely *et al.*, 1997), whereas (T_3) mainly controls the cell differentiation pathway, with strong haemoglobin accumulation

(Bauer *et al.*, 1998). The findings of present study also agreed with Korte *et al.* (1999) who explained that the partial pressure of O_2 in blood of birds decreased with low environmental temperature due to decreased respiratory rate, which would have resulted on an increased rate of erythropoeisis and consequential release of erythrocytes in to the circulation leading to increased Hb values. The untreated-10 days CST cockerels showed significant decrease in Hb concentration when compared with untreated-5 days CST cockerels. Later on, during chronic cold exposure (10 days CST) cockerels would have adapted physiologically (Hangalapura *et al.*, 2004) and thus prevented any significant change in Hb concentration.

The investigation revealed that both untreated and GB+IGN treated-NCST cockerels did not differ in Hb concentration. Even though GB was claimed to possess antianaemic function as reported by Pawar and Somkumar (2001) the property would have been hampered by the inhibitory property of tannin content in IGN. Gowri *et al.* (2001) explained that tannin content is inversely related with the haematinic effect of GB, as it reduces the bioavailability of iron.

The present study revealed that GB+IGN could not elicit any significant effect in Hb concentration during cold stress as the birds treated with GB+IGN subjected to 5 and 10 days cold stress exhibited similar response as that of its untreated counter parts.

5.11.2. Packed cell volume (PCV)

In the present study, untreated-5 days CST cockerels showed a significant increase in PCV value when compared with the untreated-NCST cockerels. Elevation in PCV could have caused by diminished plasma volume or enhanced erythropoeisis as reported by Maxwell *et al.* (1990) and Yahav *et al.* (1997). In untreated-10 days CST cockerels, the PCV value decreased significantly and reached the basal value as that of untreated, NCST cockerels, which referred to the ability of the birds to adjust to environmental variability, extremes, and cope with

the consequences of changes associated with stress as explained by Hangalapura *et al.* (2004).

The PCV value which increased significantly in untreated-5 days CST cockerels which agreed with observations of Korte *et al.* (1999) and Blake (1985) as they stated that the decrease in partial pressure of O_2 in blood of birds with low environmental temperature, due to decreased respiratory rate, would result an increased rate of erythropoiesis and consequential release of erythrocytes in to the circulation, resulting in increased PCV values. This could also be explained as an adaptogenic measure to meet increased metabolic demand on prolonged cold exposure. But the PCV decreased to reach slightly above the normal level by 10 days cold exposure in untreated cockerels.

In the present study untreated and GB + IGN treated-NCST cockerels did not differ in the PCV values. GB+IGN treated-5 days CST cockerels showed a non-significant increase in PCV and it might be due to hypohydration, and the increased erythropoeisis (Obrien *et al.,* 1998). Later, it decreased significantly to reach the PCV value as that of GB+IGN treated-NCST cockerels. Both untreated and GB+IGN treated cockerels responded similarly, indicating that the drug had no significant effect in stressed conditions.

5.11.3. Total leucocyte count (TLC)

The result of the present study revealed that untreated cockerels exposed to 5 days CST exhibited a significant reduction in TLC when compared with untreated, NCST cockerels. These findings closely agreed with an earlier report of Dabbert *et al.* (1997) who found more than 30% lower value for TLC in cold stressed birds when compared to thermoneutral controls. Results also revealed that during initial days of acute cold stress, the cockerels would have experienced stress related physiological changes that resulted in leucocytopenia. But the TLC value increased significantly on chronic cold exposure (10 days), which may be due to the innate mechanism of thermotolerance (Hangalapura *et al.*, 2004).

The GB+IGN treated-NCST cockerels showed non significant higher TLC when compared to untreated-NCST cockerels. Rekha *et al.* (2000) reported that oral administration of Brahma Rasayana that containing GB and IGN as major components, significantly increased TLC count in mice. Harikumar *et al.* (2002) also reported a significantly increased TLC in GB treated rats. Though there was a significant reduction noticed in TLC count in GB+IGN treated-5 days CST cockerels, the value increased significantly in GB+IGN treated-10 days CST cockerels. This variation was significantly higher than that observed in the untreated-5 and 10 days CST cockerels. This reinforced the immunomodulatory property as well as the leucocyte proliferative capability of the drug. This finding also agreed with Sairam *et al.* (2002) as they stated that GB had an emphasized role in immuno compromised states, through its lymphocyte prolifrerative property and also agreed with Srikumar *et al.* (2005) who stated that administration of 'Triphala' containing GB, IGN and *Terminalia belerica* significantly increased TLC in stressed rats.

From the present study, it was confirmed that GB+IGN has got a stimulatory property on stem cells of leucocytes during acute and chronic cold stress.

5.11.4. Lymphocyte (L) count

The result of the present study revealed that untreated cockerels exposed to 5 days CST exhibited a significant reduction in lymphocyte count when compared with untreated-NCST cockerels. This finding closely agreed with findings of Ben Nathan *et al.* (1976), Maxwell *et al.* (1990) and Borges *et al.* (2003) as they reported a significant decrease in circulating lymphocyte count during cold stress in chicks. The reduction in the absolute lymphocyte count (lymphocytopenia) identified in this study actually led to leucocytopenia in the corresponding groups. Hangalapura *et al.* (2004) explained an inverse relationship between lymphocytes and corticosterone release during stress.

The results of the present study indicated that cold stressed birds irrespective of GB+IGN supplementation showed a significantly lower circulating number of lymphocytes compared to NCST cockerels. These findings closely agreed with findings of Dabbert *et al.* (1997) who reported a drastic reduction in circulating lymphocytes in cold stressed cockerels. Chandratilleke *et al.*, (1994) reported that the immunosuppressive activity of high levels steroids was associated with decreased proliferative response of lymphocytes in birds.

The GB+IGN treated-5 and 10 days CST birds exhibited slightly higher lymphocyte count than the untreated counterparts. Pardue and Thaxton (1984) reported that ascorbic acid (AA) enhances lymphocyte production and lymphocyte transformation, otherwise called as immunoenhancement. Strydom *et al.* (1976) stated that AA increases body resistance to environmental stress, by altering the hormones or factors responsible for a shift in the homeostatic mechanism during stressful conditions. Scartezzini *et al.* (2006) reported that GB on processing increases the AA content by 1.28 % *w/w*. The finding of the present study also agreed with Sairam *et al.* (2002) as they stated that GB had an emphasized role in immunocompromised states, through its mitogenic property on lymphoblasts.

5.11.5. Heterophil (H) count

The study indicated that untreated-5 days CST cockerels exhibited a significant increase in heterophil count when compared with untreated-NCST cockerels and this findings agreed with the observation of Borges *et al.* (2003), who reported a significant increase in heterophil level during cold stress in male broiler chicks. Ader and Cohen (1993) stated that increased corticosterone levels might result in the significant suppression of neutrophil function and an increased number of neutrophils in stressed rats. On chronic exposure (10 days) to cold the untreated birds exhibited a further significant increase in heterophil count. The findings of the present study correlated with report by Siegel (1980) who demonstrated that stress induced increase in resistance of avian species to

bacterial pathogens due to increased number and activity of phagocytic leucocytes like heterophils and monocytes.

The GB+IGN treated-NCST cockerels showed a non significant decrease in heterophil count when compared with untreated-NCST cockerels. GB+IGN treated-5 days CST cockerels exhibited a significantly higher heterophil count when compared with the untreated-5days CST cockerels. In GB+IGN treated-10 days CST cockerels, the heterophil value decreased significantly, as that of its untreated counterparts.

The results of the present study thus revealed that upon prolonged treatment with GB+IGN during the tendency to restore to normal homeostasis could be enhanced by alleviating stress.

5.11.6. Heterophil/ Lymphocyte (H/L) ratio

The results of the present study showed that untreated-5 days CST cockerels exhibited a significantly higher H/L ratio when compared with untreated-NCST cockerels and further increase was noticed in untreated-10 days CST cockerels and these observations were in close agreement with earlier reports of Jones *et al.*, (1989). The H/L ratio has been indicated to be a good quantitative measure of stress. (Zulkifli and Siegel, 1995). The H/L ratio had been showed to be highly heritable (Altan *et al.*, 2000) and a reliable index for determining stress in poultry (Gross *et al.*, 1980). Borges, (2003) reported the trend of heterophils to increase and lymphocytes to decrease under stress in chicks.

In GB+IGN treated-NCST cockerels the H/L ratio was slightly lower than that of NCST cockerels. GB+IGN treated-5 days CST cockerels exhibited a significantly higher value when compared with drug treated NCST cockerels, and then the ratio decreased significantly on chronic exposure to cold. The ratio noticed in GB+IGN treated-5 days CST cockerels was significantly lower than that in untreated 5 days CST cockerels. In the GB+IGN treated-10 days CST

cockerels the value was significantly lower than that in drug treated-5 CST cockerels. Thus, it could be concluded that GB+IGN treatment could enhance the restoration of normal homeostasis as well as in relieving stress during acute and cold stress.

The present study also revealed that all untreated and GB + IGN treated cold stressed cockerels showed significantly higher H/L ratio when compared to control groups. However, supplementation of GB+IGN brought about an early tendency to restore to normal homeostasis of H/L ratio especially in 10 days CST cockerels.

5.12.EFFECT OF COLD STRESS ON SOME BIOCHEMICAL PARAMETERS

5.12.1. Serum protein profile:

5.12.1.1. *Serum total protein content*

The results of the study revealed that serum total protein content increased non significantly in 5 days CST cockerels when compared with untreated-NCST counterparts. This finding agreed with Dabbert *et al.* (1997) as they reported an increased serum total protein in cold stressed Northern bobwhites. Later on, untreated cockerels when subjected to 10 days CST exhibited a non significantly decrease in total protein content. The GB+IGN treated-NCST cockerels exhibited slightly higher total protein than the NCST cockerels. Nazifi *et al.* (2003) stated that total protein content was more affected in cold than in heat stress. This may be attributed to the immunomodulatary effect of GB+IGN and there by preventing the protein catabolism (Srikumar *et al.*, 2005). The GB+IGN treated-5 days CST cockerels showed a significant increase which further increased significantly on exposure to cold for 10 days. This finding of the present study coincided with the findings of Oladele *et al.* (2001) who reported that cold weather associated with low environmental temperature enhanced serum total protein content compared to high environmental temperature in pigeons. In addition, the results also agreed with the reports of Huston (1965) who correlated

an increase in serum total protein content in immature fowls during cold conditions.

The result of present study enlightened the effect of GB+IGN in preventing the protein catabolism during stress.

5.12.1.2. *Albumin content*

The results of the study showed that untreated-5 days CST cockerels exhibited a non significant increase in albumin concentration when compared with untreated-NCST cockerels and increased non significantly with chronic stress for 10 days. Albumin is being considered to be most assessable substrate for gluconeogenesis by liver (Pardue *et al.*, 1985). The GB+IGN treated-NCST cockerels exhibited a non significant increase in their albumin content when compared with its untreated, NCST cockerels. The GB+IGN treated- 5 days CST cockerels exhibited a significant increase in the albumin content, which further increased significantly on exposure to cold for 10 days. This reinforced the capability of the drug to prevent protein catabolism (Srikumar *et al.*, 2005). These results indirectly indicated that the hepatic synthesis of albumin also increased considerably during cold stress period.

5.12.1.3. *Globulin content*

In the present study when the untreated-5 days CST cockerels were compared with the untreated-NCST cockerels, there was no significant variation noticed in globulin concentration, eventhough the value decreased non significantly in 10 days CST cockerels. The GB + 1GN treated-NCST cockerels showed slightly higher value than that of untreated counterparts and this may be attributed to the immunomodulatary effect of GB+IGN and there by preventing the protein catabolism (Srikumar *et al.*, 2005).

The drug treated-5 and 10 days CST cockerels also showed a non significant decrease when compared with untreated-NCST cockerels. The present

study also revealed that serum globulin content did not differ significantly between control groups and GB + 1GN treated or untreated CST cockerels. This indicated that globulin fraction was not that much influenced either by acute or chronic stress or by GB + IGN treatment, indicating that there was no significant catabolism of globulin molecules during the period of cold stress.

5.12.2. Serum enzyme activities

5.12.2.1. *Serum lactate dehydrogenase (LDH) activity*

The results of present study pointed out that those untreated-5 days CST cockerels exhibited significantly lower LDH value than the NCST cockerels, which further increased significantly in 10 days CST cockerels. These findings did not agree with reports of Dabbert *et al.* (1997) who reported that LDH in serum of cold stressed birds were greater than that in thermoneutral or heat stressed Northern bobwhites but agreed with Davison and Lickiss (1979) who reported an increased LDH activity in cold stressed chicken. LDH isoenzymes had been found in most avian tissues and a significant increase in LDH activity represented an overall damage to the system (Kendel, 2002).

The GB + 1GN supplementation had significantly reduced the leakage of LDH from various tissues during the period of cold stress especially in 5 and 10 days CST cockerels when compared to untreated CST groups. This could be attributed to the cytoprotective effect of GB as explained by Sairam *et al.* (2002) and that of IGN as reported by Lee *et al.* (2005) as they established that extract from fruits of *T.chebula* significantly reserved the hepatotoxicity and LDH leakage into circulation from tissues, owing to the stabilizing capacity of the drug on the cell membrane permeability. Also agreed with reports of Suchalatha and Shyamaladevi (2004) as they demonstrated the cytoprotective action of IGN in rats.

5.12.2.2. *Serum creatine kinase (CK) activity*

The present study showed that untreated-NCST cockerels exhibited significantly lower value than that of GB+IGN treated-NCST cockerels. CK is mainly found in all muscles and brain tissues and it plays an important role in energy storing mechanism of the tissues (Kew *et al.*, 1971).

The GB + 1GN supplementation had significantly reduced the leakage of CK from various tissues during the period of cold stress in cockerels. This could be attributed to the cytoprotective effect of the drug, owing to the stabilizing capacity on the cell membrane permeability as explained by Sairam *et al.* (2002), Suchalatha and Shyamaladevi (2004) and Lee *et al.* (2005).

The GB+IGN treated-10 days CST cockerels showed significantly lower values of CK than that of their untreated counterparts. Hence, it was concluded that GB+IGN treatment has protective action during acute as well as chronic stress.

5.12.3. Serum electrolytes level

5.12.3.1. *Serum sodium level*

The results of the present study revealed that a non significant increase in Na concentration in acute and chronic cold stressed untreated (5 and 10 days CST) cockerels. This finding agreed with reports of Sarkar *et al.* (1981) who claimed that Na level remained unaltered during cold stress in rats. The GB+IGN treated-NCST cockerels showed a non significant increase in Na concentration than that of untreated-NCST cockerels and that value increased slightly on cold exposure especially in 10 days CST cockerels.

The findings of the present study revealed that homeostasis in maintaining normal Na level was not disturbed by cold stress neither with GB+IGN treatment or by both during cold exposure.

5.12.3.2. *Serum potassium level*

The result of the present study indicated that potassium (K) concentration increased significantly in untreated-5 days CST cockerels when compared with untreated-NCST cockerels. This finding agreed with Sarkar *et al.* (1981) who reported an increased efflux of intracellular K from red blood cells during cold stress, resulting in increased plasma K level in rats. The K level in untreated cockerels decreased significantly by 10 days cold exposure. The GB+IGN treated-NCST cockerels showed significantly higher value than untreated-NCST cockerels. Parmar and Kaushal (1982) explained that among the mineral content of edible portion of GB and IGN, K constitutes about 0.37 and 1.27 % respectively. This could be the possible reason for the slight increase in K level in the drug treated cockerels.

GB+IGN treated -5 days CST cockerels exhibited a similar response as that of its untreated counterparts indicating that the drug treatment could not elicit remarkable benefits during acute cold stress. While drug treated GB+IGN treated-10 days CST cockerels showed a significant increase in the K level compared to treated-NCST cockerels. Dabbert *et al.* (1997) explained that exposure of cockerels to extreme cold like 0°C would elevate metabolic rate to 9 fold chiefly utilizing fat reserves. When fat reserves in the body depleted, moisture content get increased as body water content being inversely proportional to fat content. In order to hold increased moisture content in the body, the total osmotic concentration of plasma had to be increased.

The findings of the present study supported this fact that by conserving more electrolytes like potassium and sodium in the serum from being eliminated during cold stress.

5.12.4. Serum C-reactive protein (CRP)

The results of the present study revealed that GB+IGN treated-NCST cockerels exhibited CRP value lower than that of untreated, NCST cockerels.

CRPs are being considered as a positive acute phase protein in poultry (Gabay and Kushner, 1999).

The untreated cockerels when exposed to cold exhibited higher value than that of GB+IGN treated counterparts. The present study showed that GB+IGN treatment in NCST cockerels could bring about a slight decrease in CRP level and this study also reinforced the cytoprotective effect of the GB+IGN as there was only moderate increase in circulating CRP levels during cold stress in GB+IGN treated cockerels, when compared to untreated CST cockerels. And these findings agreed the cytoprotective property of GB and IGN as reported by Sairam (2003) and Lee *et al.* (2005).

5.13. EFFECT OF COLD STRESS ON CERTAIN SERUM HORMONES LEVEL

5.13.1. Serum cortisol concentration

The present study revealed a significant decrease in serum cortisol level in untreated-5 days CST cockerels when compared with untreated-NCST cockerels. Even though corticosterone is the major steroid in poultry, cortisol also acts as a useful indicator of short term stress (Grandin, 1997) and it takes about 10 to 20 min to reach the peak level. After the stressors were withdrawn, the concentration dramatically declined to the normal level probably due to the short half life (1- 1 $^1/_2$ h) of cortisol (Stull and Rodiek, 2000). In the present study such an initial rise in cortisol level was not noticed. The untreated-10 days CST cockerels then exhibited a significant decrease when compared with that of untreated-NCST cockerels. These findings agreed with the reports of Luger *et al.* (2003) they reported a significantly lower level in glucocorticoid level on chronic cold exposure in broiler chickens.

The GB+IGN treated-both 5 and 10 days CST cockerels exhibited significantly lower value than its untreated counter parts. Dietary AA may inhibit steroidogenesis by elevating adrenal AA, thus limiting some of the deleterious

responses associated with stress and delay the depletion of steroid hormone precursors. Hence, the amelioration of these cold induced effects may be attributed to properties of GB+IGN either by reducing glucocorticoids output and/or protection of cells from the cytotoxic effects of adrenal steroids.

5.13.2. Serum triiodothyronine (T_3) concentration

The results of the present study showed a non significant decrease in T_3 level in untreated-5 days CST cockerels when compared with untreated-NCST cockerels and a further significant increase was noticed in 10 days cockerels. This finding agreed with Hangalapura *et al.* (2004) who stated that chronic cold stress had a significant effect on T_3 levels. Glick (1972) stated that cold environment stimulates the activity of chicken thyroid and warm environment suppresses it. Physiological adjustments to cold temperature were considered to be complex and typically involved an adjustment in thyroid metabolism (Dawson *et al.,* 1992). It is a well known fact that the thyroid hormones are mainly concerned with thermogenesis and the increase in body metabolic rate of the body. Thaxton (1978) explained that low environmental temperature related immunoenhancement, possibly mediated through hypothalamo- hypophyseal – thyroid axis.

GB+IGN treated-NCST and CST cockerels exhibited significantly lower T_3 levels than its untreated counterparts. This may be attributed to the activity of the drug and coincided with the findings of Panda *et al.* (2002) who explained the role of GB in ameliorating hyperthyroidism by decreasing the thyroid hormone concentration in mice and with Parmar *et al.* (2006) as they explained the effect of *Terminalia* extract in decreasing the level of thyroid hormones.These findings thus revealed the effect of prolonged treatment of GB and IGN in reducing the T_3 level to reestablish the normal level.

The results of the present study indicated that chronic cold exposure brought about significantly increased T_3 levels in cockerels irrespective of GB+IGN treatment. Similar observation was reported by Dabbert *et al.* (1997) in

birds under cold conditions. The results of the present study coincided with the report of Glick (1972) who reported that cold environment stimulated the activity of chicken thyroid gland while warm environment suppressed it.

5.13.3. Serum thyroxine (T_4) concentration

The results of the present study showed a significant decrease in T_4 level in untreated-5 days CST cockerels when compared with untreated-NCST cockerels. Thyroid hormones are hormones most stored in the body, a storage that could easily prevent thyroid failure for a long time, and thereby expected to maintain normal serum T_4 level (Shirpour *et al.*, 2003). In the present study, the T_4 level decreased and this finding agreed with the reports of Frank *et al.*, (1994) as they reported that during cold exposure associated with hypothermia, serum norepinephrine level increases 4-7 times their normal level and that increased norepinephrine increases the formation of endocytic cysts from droplets of follicular constituents as well as the secretion of thyroid hormones, while drastic decrease of circulating T_4 indicates the inability of T_4 to be released from its source and they concluded that hypothermia affects the secretion of T_4 directly (Shirpour *et al.*, 2003).

The T_4 level remained almost same in untreated-5 and 10 days CST cockerels and this finding agreed with Hangaalapura (2004) who found that duration of cold stress did not significantly affect T_4 levels in chickens. The present study revealed that all cockerels irrespective of GB+IGN supplementation showed significant reduction in thyroxine level. This observation coincided with reports of Cogburn and Freeman (1987) and Dabbert *et al.* (1997) as they reported that cold temperature reduced T_4 levels at the same time elevated serum T_3 levels. Subba Rao and Glick (1977) had shown that chickens secreted only half the amount of thyroxine in summer as during winter months.

The GB+ IGN treated-5 days CST cockerels exhibited almost similar T_4 level compared to untreated counterparts. GB+IGN treated-10 days CST cockerels showed a significant increase when compared with that of untreated-10

days CST cockerels and thus it could be concluded that the drug reduces T_4 level during acute cold stress while a greater tendency to restore normal level was noticed on prolonged treatment.

5.14. EFFECT OF COLD STRESS ON IMMUNOLOGICAL PARAMETRERS

Cold stress can modulate immune responses via two potential pathways., Bioenergetic pathway, if energy costs of mounting immune responses are high as proposed by Demas *et al.* (1997), and both thermoregulation and immune responses are deriving internal energy from the same source, then the increased energy requirement for thermoregulation can limit the internal energy available for the immune system. Endocrine pathway, as cold stress may alter the function of HPA axis resulting in changes in the systemic levels of hormones, which affect the immune system either directly or indirectly.

5.14.1. Haemaglutinin (HA) assay

The results of present study indicated that GB+IGN treated-NCST cockerels developed higher HA titre against SRBC when compared with untreated-NCST cockerels while the GB+IGN treated-CST cockerels developed higher HA titre than untreated-CST cockerels this may be attributed to the immunopotentiative effect of the drug (Srikumar *et al.*, 2005).

The cold induced immunoenhancement observed in the study agreed with the report of Subba Rao and Glick (1977) as they postulated hypothalamo-hypophyseal-thyroid axis theory for immunoenhancement at 15°C in birds. While, Regnier *et al.* (1980) found little effects of acute cold stress on antibody titres to sheep red blood cells in both broilers and layer chickens.

5.14.2. Haemolysin (HL) assay

The results of present study indicated that GB+IGN treated-NCST cockerels developed higher HL titre against SRBC when compared with untreated-NCST cockerels and the GB+IGN treated-CST cockerels developed

higher HL titre than untreated-CST cockerels. Thaxton (1978) reported that when chickens were subjected to cold temperature, humoral immune response was enhanced. More over cold stressed groups irrespective of GB+IGN treatment exhibited the peak response one day ahead when compared to other groups. These results reinforced that GB+IGN supplementation during cold stress enhanced humoral immune responses in chicken against a known antigen.

5.14.3. Serum mercaptoethanol resistant antibody (Ig G) level

The results of present study indicated that the titre value of IgG produced against SRBC in GB+IGN treated-NCST cockerels was higher than the titre in untreated-NCST cockerels. It was also observed that the preformed IgG titre against SRBC in GB+IGN treated cockerels whether cold stressed or not, was found to be 2 ½ times more than untreated cockerels on the 5^{th} day after immunization and this may be attributed to the immunopotentiative effect of the drug (Srikumar *et al.*, 2005). Findings of the present study did not agree with observations of Subba Rao and Glick (1977) who reported that cold treatment significantly lowered IgG level, which indicated cold induced changes in the metabolic activity of antibody forming cells. These findings reconfirmed the immunopotentiative action of GB+IGN supplementation in cockerels.

5.14.4. Serum mercaptoethanol sensitive antibody (Ig M) level

The findings of the present study indicated that cold stress could bring about any appreciable increase in IgM tire in untreated and GB+IGN treated cockerels. Hence, the result of the study positively correlated with the reports of Subba Rao and Glick (1977) as they reported that absolute turn over of both IgG was increased by cold exposure with out accompanying decrease in the level of IgG in pullets. They reported a significant enhancement of IgM due to cold treatment. Collectively, this data suggested that when cockerels are subjected to either continuous or acute exposures to a cold temperature, humoral immunity was enhanced. Apparently, this enhancement was related to increase circulating levels of IgG. Thaxton (1978) explained this effect as low environmental

temperature mediated immunoenhancement, possibly mediated through hypothalamo- hypophyseal –thyroid axis.

5.15. EFFECT OF COLD STRESS ON RELATIVE WEIGHT OF ADRENAL AND SPLEEN

5.15.1. The relative adrenal weight

The results of the study suggested that cold stress brought about significant reduction in relative adrenal weight in cockerels when compared to GB+IGN treated and control cockerels. This finding did not agree with Nachankar *et al.* (1998) as they reported increased weight and reduced AA content in adrenal glands of acute cold stressed rats. The findings of the present study indicated that the GB+IGN treatment in CST cockerels helped to maintain the adrenal weight unaltered during the period of acute as well as chronic stress and this may be attributed to the protective property of the drug.

5.15.2. The relative spleen weight

The present study revealed that GB+IGN treatment brought about a significant increase in relative spleen weight in all cockerels irrespective of cold exposure compared to untreated cockerels. The increased spleenic weight encountered in this study could be attributed to the immunopotentiating action of AA resulting in appearance of new germinal centers in spleen. This observation reconfirmed an earlier report of Pardue *et al.* (1985) who found that AA supplementation in chicks resulted in larger bursa and spleen weight. This observation was almost similar to the response shown by heat stressed cockerels and the immunopotentiating action of GB+IGN treatment was thus reconfirmed.

5.16. EFFECT OF COLD STRESS ON ADRENAL HISTOLOGY

Histological study revealed that there was no noticeable change observed in the adrenals of either untreated or GB+IGN treated cold stressed cockerels. Unlike in heat stress, exposure to cold in cockerels did not affect

histomorphology of adrenal glands and this finding closely agreed with Pacak and Palkovits (2001) who stated that adrenal gland responds differently to the various stressors.

5.17. EFFECT OF COLD STRESS ON ANTIBODY FORMING CELLS

5.17.1. Jerne's plaque assay

The result of present study demonstrated that anti-SRBC PFCs in GB + IGN treated-NCST cockerels were significantly higher than untreated-NCST controls. This finding agreed with Fernandes (2000) and Fujiwara *et al.* (1999) they reported increased PFC during stress. While those cockerels exposed to chronic stress exhibited decreased PFC count and this finding agreed with Bhatnagar *et al.* (1996) reported PFC decreased with chronic and intermittent cold exposure. The over all improved response showed by GB+IGN treated cockerels (both CST and NCST) over the untreated cockerels (both CST and NCST) could be attributed to the stimulative effect of the drug on the spleenic cells to produce antibodies against RRBC.

5.17.2. Rosette – forming cell (RFC) immunocytoadherence assay

The present study revealed that the number of anti-RRBC RFCs encountered were higher than number of anti-RRBC PFCs. This observation coincided with the earlier report of Tufveson and Alm (1975); Thursh *et al.* (1972) and Dagg *et al.* (1997). This study confirmed that GB+IGN supplementation improved anti-RRBC- RFCs in GB+IGN treated cockerels over untreated cockerels irrespective of cold exposure. It was also found that GB+IGN treated cockerels exhibited a significantly greater per cent of RFCs against RRBC when compared to untreated cockerels. Thus cold exposure along with GB+IGN treatment stimulated spleenic cells to produce anti-RRBC- RFCs at an early date when compared to untreated-NCST cockerels (Tufveson and Alm, 1975). This study also established that RFC assay was more sensitive than PFC assay during cold stress conditions in assessing humoral immune status in birds.

Summary

6. SUMMARY

From the present investigation it can be concluded that supplementation of GB+IGN under heat and cold stress in cockerels could bring about marked alleviation of stress. Heat stress brought about significant changes on certain haematological, biochemical, hormonal and immunological response. In general, the severity of alternations on the above mentioned parameters could be markedly reduced by GB+IGN supplementation, which could be attributed to the antistress property of the drug.

Heat stress (40 $\pm 1^{\circ}$ C and RH 60-70 percent for 10 days @ 4h/day) in cockerels brought about a moderate hyperthermia, which could be due to accelerated muscular work associated with increased respiratory and cardiovascular activity as well as increased metabolic heat production. The mild dehydration that the cockerels encountered during heat stress brought about significantly higher values in haemoglobin (Hb) and packed cell volume (PCV). During continued heat exposure as a measure of adaptogenesity, cockerels exhibited Hb and PCV in par with controls. The present study revealed that GB+IGN could not elicit any significant effect in Hb and PCV concentration during heat stress in cockerels as the birds treated with GB+IGN subjected to 5 and 10 days heat stress exhibited similar response as that of its untreated counter parts.

From the present investigation it could be established that heat stress brought about severe leucocytopenia associated with significant reduction in absolute lymphocyte count and a relative increase in heterophil count. However, GB+IGN @ 2.0 g/kg bw for 20 days supplementation brought about a rapid restoration in total leucocyte count (TLC) to normal homeostasis. The variation seen in TLC in heat stressed (HST) cockerels could be attributed to the continued activation of hypothalamo – hypophyseal – arenal (HPA) axis and resultant release of adrenal steroids which brought about involution of lymphoid organs.

In the present investigation, it was found that no significant change were noticed associated in GB+IGN treated-HST group's serum total protein, albumin and globulin explaining the protective property of the drug on blood proteins from being metabolized. The supplementation of GB+IGN in HST groups had significantly reduced the leakage of lactate dehydrogenase (LDH) and creatine kinase (CK) into circulation from tissues, owing to the stabilizing capacity of the drug on the cell membrane permeability.

From the present study, it could be established that GB+IGN supplementation did not result in the marked elevation of serum C-reactive protein (CRP) and the drug treated exhibited an early tendency to return to the basal line of serum cortisol values, thereby reinforcing its antistress nature during the period of heat stress.

The hyperthermia dependent increase in T_3 concentrations observed in study could be due to the thermogenic function associated with this hormone. The GB+IGN treated, HST cockerels showed similar pattern of separation of serum proteins with that of control chicken whereas untreated HST cockerels showed some deviation from the normal pattern.

The GB+IGN supplementation did not allow adrenals to loose its weight during heat stress and at the same time markedly increased in the size and weight of spleen owing to its immunopotentiative property. There was only moderate level of fibrotic invasion in the adrenals of GB+IGN treated, HST cockerels when compared to its untreated counterparts.

Heat stress showed a low profile of humoral immune response indicated by low anti-SRBC haemolysin (HA), Haemolysin (HL), IgG and IgM titres in untreated, HST cockerels when compared to GB+IGN treated counterparts. This again confirmed the immunopotentiative action of GB+IGN supplementation in HST cockerels. Similarly, treated cockerels exhibited more spleenic cells that produce antibodies against RRBC, which were confirmed by PFC and RFC assays.

The present study indicated that exposure of cockerels to cold (8 ±1° C and RH 40-50 percent @ 4h/day) stress, resulted in an average loss of 4% bw at the end of the stress period in cold stressed birds suggested that fat reserves were partly used to fuel the metabolic demands despite the availability of feed.

A significantly higher PCV value on 10 days cold stressed birds could be explained as an adaptogenic measure to meet increased metabolic demand on prolonged cold exposure. Results also revealed that during initial days of acute cold stress the cockerels would have experienced stress related physiological changes that resulted in leucocytopenia. However, the combined treatment of GB+IGN brought about an early restoration in TLC of GB+IGN treated-10days CST cockerels.

The results of the present study indicated that all cold stressed birds irrespective of GB+IGN supplementation showed a significantly lower circulating number of lymphocytes compared to control groups. The study indicated that heterophil count in all cold stressed untreated and GB+IGN treated cockerels increased significantly and supplementation of GB+IGN brought about an early tendency to restore to normal homeostasis of H/L ratio especially in 10 days CST cockerels.

The present study indicated that the significant increase in serum total protein content of cold stressed cockerels irrespective of GB+IGN treatment was absolutely due to increase in the albumin content of the serum. LDH activity in cockerels reared under thermoneutral conditions was significantly higher than cold exposed cockerels, it would be assumed that cold environment reduces the leakage of LDH from organs in to the blood, indicating minimal damage to the various organs compared to hot environment. An increased activity of serum CK encountered in the study could be due to more discharge of CK from skeletal muscles, especially those associated with involuntary shivering thermogenesis during cold exposure. The present study indicated that serum potassium level of

all cockerels exposed to cold stress irrespective of treatment with GB+IGN exhibited significantly higher values compared to controls.

From the present study, it could be established that GB+IGN supplementation did not result in the marked elevation of serum C-reactive protein (CRP) and serum cortisol values reinforcing its antistress nature during the period of cold stress. The results of the present study indicated that during cold exposure brought about significantly increased T_3 levels in cockerels.

Irrespective of GB+IGN supplementation adrenals exhibited weight loss while markedly increased size and weight of spleen during cold stress was observed. Unlike in heat stress exposure to cold in cockerels did not affect histomorphology of adrenal glands.

Cold stress showed a low profile of humoral immune response indicated by low anti-SRBC haemolysin (HA), Haemolysin (HL), IgG and IgM titres in untreated, CST cockerels when compared to GB+IGN treated counterparts. This again confirmed the immunopotentiative action of GB+IGN supplementation in CST cockerels. Similarly, treated cockerels exhibited more spleenic cells that produce antibodies against RRBC, which were confirmed by PFC and RFC assays.

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EFFECT OF GOOSEBERRY (*Emblica officinalis*) AND INDIAN GALL NUT (*Terminalia chebula*) ON THE IMMUNE RESPONSE IN COCKERELS UNDER INDUCED HEAT AND COLD STRESS

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ABSTRACT

The study was conducted with an objective of finding the physiological and immunological variations that could be brought about with heat /cold stress in cockerels and the role of Gooseberry (GB) and Indian gallnut (IGN) supplementation as an antistress. Gramapriya cockerels of 1kg bw (3-4 months) were subjected to heat (40 $\pm 1^{\circ}$ C and relative humidity (RH) 60-70 percent) and cold stress (8 $\pm 1^{\circ}$ C and RH 40-50 per cent) each for 4h/day in a controlled environmental chamber (floor space 875 cm^2 /bird) for a maximum of 10 days and the controls were reared randomly under ambient temperature of $30 \pm 1^{\circ}$ C and RH 65 percent. GB+IGN supplementation was done @ 2.0 g/kg for 20 days (prior to and during the period of heat/cold stress).

To a certain extend alternations in haematological parameters such as haemoglobin, packed cell volume, H/L ratio, biochemical parameters such as serum total protein, albumin, globulin, C-reactive protein, electrolytes like sodium and potassium, enzymes like lactate dehydrogenase and creatine kinase, cortisol, triiodothyronine (T_3), thyroxine (T_4) could be reversed by GB+IGN supplementation during heat stress.

In the present study, the haemagglutinin (HA), hemolysin (HL), IgG and IgM titres and the spleenic antibody forming cells (plaque forming cells) and rosette forming cells (RFC) against known antigen were studied. The results indicated that GB+IGN supplementation not only maintained the preformed antibody titre but also improved the humoral immune response against a challenged antigen during the period of heat stress.

In the present study, it was found that during cold stress, the GB+IGN supplementation could bring about an early tendency to restore the normal homeostasis of haematological, biochemical, and hormonal parameters. Cold stress resulted in a low profile of humoral immune response indicated by low anti-SRBC haemolysin (HA), Haemolysin (HL), IgG and IgM titres in untreated,

CST cockerels when compared to GB+IGN treated counterparts, which showed better tires during cold stress. Similarly, treated cockerels exhibited more spleenic cells that produce antibodies against rat red blood cells. Thus, the immunopotentiative property of GB+IGN was reconfirmed and that the drug supplementation stimulated the humoral arm of immunity in cold stressed cockerels.

Results of the present study indicated that combined supplementation of GB+IGN @ 2.0 g/kg bw in poultry could augment the humoral response during heat and cold stress.